VIRUSES 1950

Edited By

M. DELBRÜCK

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Proceedings of a conference on the similarities and dissimilarities between viruses attacking animals, plants, and bacteria, respectively. Held at the California Institute of Technology, March 20-22, 1950.

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CONTENTS

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FOREWORD

Animal and plant viruses are of great practical importance because they are disease producing agents. As such they are of interest to pathologists, and research in this direction is heavily sponsored by medicine and by animal and plant industry. This research aims directly at the control of specific virus diseases, but notwithstanding the expenditure of great effort practical success has been very limited. It is true that we have learned something about the natural history of a number of the disease producing agents and that some of them have been isolated and partially characterized but this is not enough to suggest remedies. What is needed is an understanding of the behaviour of viruses within their hosts. How do the viruses invade host cells, how do they multiply, how do they interrupt and modify the normal functions of the host cell? These are the problems of virus research which tie this research into the great stream of modern biology aiming at the analysis of cellular functional organization.

The conference which gave rise to the proceedings here presented arose from the desire to bring the men who work on the three great groups of viruses, those which attack animals, plants, and bacteria, respectively, into one room and to discuss whether and to what extent our respective charges can be brought under one hat. We felt that such a move could be profitable only if each of us did some considerable home work of a double nature, namely, that of preparing an intelligible statement concerning his own specialty and that of studying the prepared statements of the others. To set a background to the general approach an essay by Luria, which had just been submitted for publication in Science,[1] was circulated among the prospective participants. The preliminary statements were also circulated, and were studied and discussed at home and en route by smaller groups out here and by those who had arranged to make the long trip West together. The conference was in fact proceeding for quite some time before it met as a whole, and it continued for some of us for three days after the meeting around camp fires in Death Valley. When it met as a whole a great deal of formality could be dispensed with. The authors of the preliminary statements confined themselves to briefly recalling the highlights of their papers and then laid themselves open to questions from the floor, of which there were many. Most of these questions were of such a nature as to suggest emendations, additions, or deletions. Rather than print these discussions it was left to each author to incorporate into his paper whatever profit he had derived from them.

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[1]Grateful acknowledgement to the Editor of Science is hereby made for permission to reprint this essay. It has since appeared in slightly modified form in the May 12 issue of Science (*111*, 507, 1950).

Thus, many of the critical remarks appear in this volume only as reflected in the minds of the authors to whom they were addressed.

A few of the remarks contributed essentially new material or a new point of view, and in these cases the speakers later prepared the text to be included here.

Four reports submitted for the conference are not included in the present volume. Dr. Isabel Morgan Mountain had written a very interesting report on immunity to mammalian viruses. It was felt however that the phenomena discussed by Dr. Mountain, being entirely specific to mammalian hosts, fell too little within the scope of this book whose chief purpose it is to compare the different virus groups. Dr. C. W. Bennett had written a report on interference in plant viruses, but preferred not to have his paper included, since he is now writing a review article on this subject which is scheduled to appear in next year's Annual Review of Microbiology, and will embody results of experiments now in progress. Mr. Bawden's discussion remark on interference fills this gap in some measure. Drs. S. G. Wildman and R. D. Owen discussed their current work on the relationships between host protein and virus protein in tobacco mosaic disease, but wished to postpone publication of this study until certain crucial experiments now in progress are completed. N. W. Pirie, although not able to attend the meeting, very kindly contributed a paper entitled "The biochemical approach to viruses." At the request of the editor, Messrs. Bawden and Pirie substituted for this paper the one here included, which is more explicit in presenting the data to which Mr. Pirie's arguments referred.

An explanation should be added about the sequence in which the material was discussed and the sequence in which it is here presented. One might almost say that if we knew a logical order of presentation the conference need not have been held. It is just the uncertainty of the homology and of the interpretation of many of the facts which was the topic of discussion, and, needless to say, much of this uncertainty still remained at the end of the conference. At the conference we did make, nevertheless, a timid attempt at a logical sequence, working our way from the stage of adsorption of a virus, through the stage at which interference may be presumed to occur, to the more intimate stages of virus reproduction, where chemistry, immunology, and genetics are trying to blaze their respective trails, not knowing whether or where they will meet. We moved back and forth freely among the three groups of viruses, permitting free rein to the demands of the subject matter.

In presenting the material in printed form we have chosen a different sequence. Luria's paper is presented first since it is more

comprehensive and theoretical in scope than any of the others. It served as preamble to those who wrote the other papers and may well serve as such also to the readers of this book. The other papers and the major discussion remarks then follow in alphabetical order of their authors. It was felt that the book may be most useful for reference purposes and that this arrangement will make it easiest to find a given paper. Finally, we have put at the end, as a kind of appendix, a syllabus on procedures, facts, and interpretations in phage. This was prepared by the phage group at Caltech, and is intended as a guide to the phage literature of the last years. A short paper presented at the conference by W. Weidel on his recent findings on receptor spots of bacteria has been inserted into the syllabus as section 17a (p. 119). This syllabus has been read by most of those whose work has been cited and their comments have been taken to heart in preparing the revised version here printed. No general criticism was expressed in any of these comments with one exception. Dr. S. S. Cohen felt that the syllabus did not give proper weight to the biochemical approach, to which he himself has so pre-eminently contributed, and that this failure of a proper appreciation of the biochemical results had led to a false appraisal of the present situation. While the authors of the syllabus do not accept this criticism they would like to draw particular attention to the recent review article by Dr. Cohen in the Bacteriological Reviews, to which reference is made in the syllabus, and which should be considered as a complementary report, giving particular emphasis to the biochemical approach to phage.

We would like to ask for special indulgence from the readers of this book on two scores. First, there are many who could have made important contributions but were not invited simply because we wanted to have an informal conference and therefore a small group. Thus, the readers should not expect a comprehensive coverage of all virus problems in this book. Second, when the participants were asked to make their preliminary written statements, they were asked to do so with the specific purpose of enlightening the fellow participants, and not a wider public. It was only after the manuscripts had been received and distributed that the idea of revising and printing them began to be considered. Originally the papers contained no references to the literature, and only a few were added later. The reader should keep in mind that these papers are not formal review articles. They are personal evaluations of the virus problems as they appeared to some of us in 1950.

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M. Delbrück.

BACTERIOPHAGE

An Essay on Virus Reproduction

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In a discussion of the mechanisms involved in virus reproduction, it is well to start with a critical revision of concepts and definitions, because some of the ideas conceived in other fields have carried into virology implications not justified by the methodology of virus research.

The term "virus" itself can be operationally defined as an *exogenous submicroscopic unit capable of multiplication only inside specific cells.* This definition gives a methodological unity to the field of virology and, by leaving ambiguous two borderline fields—that of obligate parasitic microbes, on the one hand, and that of protoplasmic components transmissible by graft only, on the other hand—suggests some of the possible natural relationships of viruses.

The concept of reproduction requires closer scrutiny. What we observe is the appearance of increased virus activity, associated with an increased number of specific material particles, in a population of virus-infected cells. Virus is produced by the only observable entity, the virus-infected cell, and the mechanism intervening between infection and appearance of the new virus activity cannot be postulated by analogy. In many minds the terms "reproduction" and "self-reproduction" are connected with the idea of increase in size followed by division. Closer scrutiny reveals that increase in size followed by division is bound to be an epiphenomenon of some critical event of reproduction, which must involve point-to-point replication of some elementary structures responsible for the conservation of specificity from generation to generation. Thus, in dealing with cell growth and division we trace the critical event to gene and chromosome duplication. Even a bag of enzymes could only grow and multiply by duplication of discrete enzyme molecules, which can hardly be supposed to grow individually in size and then split. In a repeat, crystal-like structure, such as has been suggested for rod-shaped particles of plant viruses (2), the elementary repeated unit must be replicated. In other words, all growth and reproduction should ultimately be traceable to *replication of specific chemical configurations by an essentially discontinuous appearance of discrete replicas.*

One of the first tasks in virus research is to uncover the relation of the virus particle, as we know it in the extracellular state, to what is replicated inside the infected host. Misunderstandings may arise, however, if we fail to distinguish between replication and the more general category of chemical synthesis. There is something peculiar to homologous replication that sets it aside from other types of synthetic re-

actions. The replication of specific biological units must involve the building of complex specific molecules or molecular aggregates, the only permissible limitation to identity of model and replica being the production of "mutated" structures, that is, of modified elements replicated in the modified form. The indispensable presence of the initial model (gene, virus) indicates that this model plays a role in replication, but this role is by no means an obvious one. The model might carry within itself all the enzymes needed for its own synthesis from specific building blocks, or it might act as a directive pattern for synthesis, according to which building blocks are assembled by synthetic enzymes not pertaining to the model itself [this may require a two-dimensional unfolding of the model, to allow point-to-point replication followed by separation of the newly formed unit (33)], or it might function as a directive pattern for folding a pluripotential macromolecule into a specific tridimensional replica, possibly with the intervention of a negative template, by analogy with Pauling's theory of antibody formation (31).

The study of virus reproduction constitutes one of the best approaches to bridging the gap between growth and replication. I shall deal primarily with the study of bacterial viruses as exemplified by the system of the "T" phages (T1-T7) active on *Escherichia coli* strain B (5). Reproduction takes place in a short "latent period" (13 to 45 minutes for different viruses under standard conditions) between the infection of a bacterial cell and its dissolution or lysis, with a rise in phage activity traceable to liberation upon lysis of large numbers of specific phage particles. The number of infected cells, the number of phage particles infecting each cell ("multiplicity of infection"), the time between infection and liberation and the amount of virus liberated by each cell can be determined accurately (7). Moreover, the infecting virus may be "labeled" with easily recognizable properties arising by mutations (23, 15). Our problem is the following: how are the newly produced phage particles related to the infecting particles?

On the one hand, the continuity between the two is evidenced not only by the general specificity of reproduction, but also by the observation that mixed infection of a bacterial cell with two closely related phages, such as T2 and its mutant T2r, causes a mixed yield of both infecting types in proportions similar to the ones in the infecting mixture (15). The continuity of virus material after infection is also proved by irradiation experiments, which show that the radiation sensitivity of phage inside a bacterium remains the same as that of free phage for a few minutes after infection, indicating that the total amount of radiation sensitive material remains unchanged (26, 21).

On the other hand, there is ample evidence of a deep-reaching alteration of the virus after infection. Doermann, breaking open phage-infected bacteria at different times after infection, found that for several minutes no phage activity could be recovered, and only around the middle of the "latent period" do the first active particles make their appearance (8). A similar conclusion had been reached in my laboratory from the study of the effect on phage-infected bacteria of the dye proflavine, which apparently stops phage production, but allows bacteria to lyse and liberate whatever particles were already present when the dye was added (12). Here, too, no active phage is liberated by cells to which acriflavine was added during the first period. Thus, there is an "eclipse" of recoverable phage activity between the disappearance of the infecting particles and the appearance of new ones. To the relation between the two I shall return later.

Another line of evidence indicating that the final particles are not a direct product of reproduction of the infecting particles *as such* is the occurrence of complex interactions revealed by experiments with mixed infection. Mixed infection of bacteria with pairs of unrelated phages, such as T1 and T2, or T1 and T7, gives "mutual exclusion": only one type of particle is liberated by each bacterium and the infecting particle of the other type is lost, again suggesting an alteration of the infecting particles (7, 4). Phages T2, T4, and T6, however, form a related group and mixed infection of a bacterium with two of them gives rise to a mixed yield. If the two infecting types differ in a character whose alternative forms can manifest themselves in both types—for example, T2r$^+$ and T4r—they give progeny containing, besides the parental types, some new types also, which result from a recombination of the alternative characters r and r$^+$ present in the parental types: T2r$^+$, T2r, T4r$^+$, T4r. This fundamental result, obtained by Delbrück and Bailey (6), was greatly extended by Hershey and Rotman (16, 17) who, studying recombination among different mutants of phage T2 infecting the same host cells, showed that recombinant types occur with definite specific frequencies for different characters, suggesting a localization of the hereditary properties of bacteriophage in discrete material determinants. We can then, at least formally, interpret recombination experiments according to the model of a phage particle composed of a number of discrete recombinable genetic units, whose number is probably quite large, of the order of 100 or more.

Another kind of interaction is "multiplicity reactivation," which consists in the production of active bacteriophage inside bacteria infected with *two or more* particles of some bacteriophages previously

exposed to ultraviolet light and "inactivated"—in the sense that infection of a bacterium with *one* such irradiated particle, while killing the bacterium, would not cause any phage production (24, 25). For reactivation to take place, the inactive infecting particles must be of the same type or of genetically related types (T2, T4, T6). This can be interpreted on the basis that reactivation is due to replacement of damaged portions or units of the genetic material of one infecting particle by homologous undamaged portions supplied by the other particles, by the same (unknown) mechanism responsible for the genetic recombinations discussed above. This interpretation of multiplicity reactivation leads to specific expectations concerning the frequency with which bacteria infected with inactive phage particles should produce active phage. On the one hand, the greater the dose of radiation, the smaller should be the frequency of the bacteria that receive two or more particles which can successfully supplement one another, because of more frequent damage in homologous genetic units. On the other hand, for a given dose of radiation, the frequency of bacteria producing active phage should increase with increasing "multiplicity of infection," since this increases the fraction of bacteria that contain mutually supplementing groups of inactive particles. Both expectations are borne out by experiment, and the results agree reasonably well with quantitative expectations derived from a mathematical rationalization of the genetic hypothesis of reactivation. This hypothesis, however, should be considered simply as a working hypothesis until it is substantiated by independent evidence; for the time being it rests mainly on analogy and on a mathematical analysis involving several unproved assumptions. Dulbecco (10, 11) has recently discovered in my laboratory that ultraviolet inactivated phage attached to its host bacterium can be reactivated by exposure to visible light ("photoreactivation," 20). The results of this work may affect, in a way that is not yet clear, the interpretation of multiplicity reactivation as well.

Be this as it may, the interactions among phage types in mixed infection indicate that in phage reproduction specificity is perpetuated not as a whole, but subdivided into discrete units; we must then look upon these units as the elements whose specific structure is replicated. This result alone does not prove, however, that the units are replicated *separately:* one could imagine that, after the initial "eclipse," each new phage particle is produced as a whole and that all recombinations result from late interactions among the newly formed particles. To gain information on this point let us return to the experiments on the kinetics of intracellular phage production.

We saw above that active phage particles appear only around the

middle of the latent period; afterwards, their number increases at an approximately linear rate, as shown by Doermann's breakage experiments (8). Using mixed infection with different mutants, as in Hershey and Rotman's experiments (17), Doermann has recently proved that the very first crop of active particles to appear inside infected bacteria already comprehends the same variety of parental and recombinant types that are present in the final yield, and in the same proportions (9). This indicates that the interactions leading to a recombination must take place in the early stage of infection, at a time when no fully active particle can be recovered. As in a well-ordered kitchen, the basic cooking appears to have been done before the first course is served. It seems indeed a reasonable working hypothesis to assume that the active phage particles, which appear at a linear rate in the late phases of intracellular growth, are the end products of reproduction and may, therefore, play no role in further phage production.

The complexity of the interactions that occur in the early period of phage infection is evidenced by several observations. Multiparental, rather than only biparental reproduction, must be assumed if we want to account quantitatively for the multiplicity reactivation of ultraviolet inactivated phage by the genetic recombination hypothesis (25). Triparental reproduction was directly proved by Hershey and Rotman using mixed infection with three different T2 mutants (16). For these and other reasons, all of which suggest repeated and independent contributions of each infecting particle to the formation of several offspring, I have proposed the hypothesis (24) of an independent replication of the genetic units composing the phage, followed by their reorganization into complete, mature phage particles. It should be clearly remembered that no independent evidence for this mechanism of independent reproduction of units has as yet been obtained.

This seems to be as far as we can go at present in analyzing phage production from evidence supplied by the end products. The biochemist has recently thrown some interesting light on phage reproduction, approaching it from the direction of the non-specific building blocks. The main results (see 3), obtained by determination of total protein and nucleic acids in infected bacteria and by isotope techniques, indicate that the material of the phage particles—which consist only or almost only of protein and desoxyribonucleic acid (DNA)—derives in the greatest part from compounds assimilated from the medium after infection. The rate of assimilation of these new materials is similar to the rate of synthesis of bacterial protoplasm in non-infected cells immediately before infection. This suggests that the pre-existing

synthetic enzymes of the bacterium are responsible and rate-limiting for the formation of the building blocks for phage synthesis. DNA synthesis immediately precedes and parallels the appearance of active phage particles and fails to take place in bacteria infected with inactive, nonreactivated phage, which suggests that DNA may be involved mainly in the final steps of the "baking" of active particles. Infection with ultraviolet inactivated phage not leading to active phage production is, however, accompanied by the production of some ultraviolet absorbing material, as yet unidentified (28). It would be rash to interpret these abortive syntheses as due to the activity of portions of the infecting phage particles that are not damaged by ultraviolet radiation.

Failure of phage infected bacteria to produce specific bacterial components, as distinct from phage substance, is shown by the elegant experiment of Monod and Wollman (30) on the absence of adaptive enzyme formation in phage-infected bacteria. A similar failure of enzymatic adaptation has been observed in bacteria infected by ultraviolet inactivated phage under conditions in which no reactivation occurs (27).

A rationale for the suppression by phage infection of specific bacterial syntheses is suggested, in the light of current theories of gene action, by cytological observations (28). The first result of infection of a bacterium either with active or irradiated phage T2 is a rapid disruption of its nuclear apparatus represented by the Feulgen-positive "chromatin" bodies. In the case of infection with active phage, nuclear disruption is followed by the appearance of a granular type of chromatin which probably represents the new phage itself, as indicated by the failure of this new chromatin to accumulate either after infection with inactive, nonreproducing phage, or after infection with active phage in certain abnormal bacterial strains which upon lysis fail to produce any active phage. These observations suggest that the suppression of synthesis of specific bacterial components in a phage infected bacterium results from a disruption of the genetic apparatus of the bacterium and its replacement with the genetic apparatus of the virus, resulting in viral rather than bacterial specificity of the protoplasm newly synthesized by the available bacterial enzymatic machinery. The disruption of the genetic apparatus of bacteria infected with inactive phage explains the failure of these bacteria to undergo any further multiplication.

According to this hypothesis, the virus does not only introduce into the host bacterium an *additional* organizer of specificity, but a com-

pletely *predominant* one, in what could be called parasitism at the genetic level. In the so-called "lysogenic" strains of bacteria, which carry and occasionally liberate phage (29), the genetic patterns of host and virus may coexist and function side by side in genetic symbiosis. The difference between phage infection followed by death of the host and phage infection followed by lysogenicity may thus be interpreted as a difference in compatibility relations between the genetic materials of virus and host. The compatibility in lysogenic systems may be more or less stable, and its changes may be connected with the sporadic character of phage liberation by lysogenic bacteria (29).

Stretching the available evidence, one may construct the following picture of the reproduction of a bacteriophage such as T_2: Infection produces a disruption of the genetic organization of the host and a change in the organization of the infecting virus leading to the formation of a new unit system, the *virus-infected cell*, containing the existing enzymatic machinery of the host and, superimposed upon it, a genetic pattern derived from the virus and directing the synthesis of virus material from non-specific building blocks. This genetic pattern is resolved into a number of discrete, more or less independent units, the genetic determinants of the virus. The process of formation of the new virus is such that it allows for complex reorganizations to take place and results in the appearance of a population of virus particles which represent the end products of the process as a whole.

This picture, which admittedly has a heuristic rather than descriptive function, presents several major gaps. First, there is a time gap between the disappearance of the initial virus and the appearance of the mature virus. Second, there is a chemical gap between the aspecific building blocks and the final specific nucleoprotein particles. Third, we have a genetic gap between the determinants of the phage and the phage particle, the former being responsible for the inherited specificities, the latter being the carrier of infectivity and, therefore, the only operationally definable unit in the extracellular state. Finally we have a technological gap, in our ignorance of the enzymatic machinery involved in the synthesis of phage from the newly assimilated building blocks. I do not emphasize these gaps in a spirit of pessimism, since it is clear than they involve phases of biological replication about which no biologist possesses any information. The very fact that these gaps can clearly be visualized and delimited in phage analysis suggests that they may be filled more easily by work on bacteriophage than on other biological systems.

How far results of phage research can throw light specifically on the events of other virus infections, we do not know. Virus-host relationship may include systems so different that the only similarities to be postulated *a priori* are those implied in our definition of "virus." Nevertheless, the picture of reproduction emerging from phage research is likely to bear instructive similarities to other virus infections. Disappearance of recoverable virus activity following infection of a host cell is of general occurrence. Disruption of the genetic apparatus of the host is certainly not general, since cells infected by any one of several plant or animal viruses can still grow and divide. Changes in the synthetic pattern of virus infected animal cells similar to those of phage infected bacteria, however, have been recognized (19).

Influenza virus in the chorioallantoic membrane of the chick embryo behaves very much like bacteriophage in a culture of a susceptible bacterium, with discrete cycles of intracellular growth and liberation, mutual exclusion in cells infected by two virus strains, and other similarities (13); a genetic analysis of this situation would be very desirable. As virus reproduction is apparently more on a level with the reproduction of the genetic material of other cells than with the reproduction of the whole cell itself, it does not seem rash to assume that in all virus infections the material carrying virus activity will be found to be differently organized in its intracellular, replicating, "dynamic" state than in the extracellular, "static" condition. This makes it unlikely that even the most careful and painstaking work on the physical properties of extracellular virus particles (22, 32), although very interesting from other points of view, can throw much light on the fundamental problem of virology: virus reproduction. The limitation appears to be an operational one—the alteration, upon infection, of the very properties that the physicochemist analyzes.

In contrast, the limitation of chemical studies on virus-infected cells is merely a technological one—the inadequacy of present day organic chemistry to deal with the level of organization at which biological specificities are encountered. A sharp refinement of the chemical tool is available, however—immunochemistry. Viruses are good antigens and are in general completely distinct serologically from the uninfected host cells. Can virus specificity be traced serologically during virus reproduction, even in the absence of demonstrable virus activity, to reveal to us the "intermediates" of virus synthesis? The beautiful work of Hoyle (18) and Henle (14) on the complement-fixing antigens in the various phases of influenza virus infection shows that these antigens, which carry virus specificity without virus activity, increase in amount *before* virus activity appears. Similar methods now being ap-

plied to the study of the early phases of phage production should yield very valuable information.

What will the "intermediates" of phage reproduction, if any, be like? Will they disclose the structure of the hereditary material represented by the postulated genetic units of replication? The recent discovery of an osmotic membrane around the phage particle (1) suggests that the latter may consist of both genetic and non-genetic specific materials; caution will be necessary in distinguishing between the two. Nevertheless, it is not unreasonable to hope that this line of work will bring us one step closer to our ultimate goal, the identification of the elementary "replicating units" of biological material and the clarification of their mode of reproduction.

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Addendum

THE DISTRIBUTION OF SPONTANEOUS PHAGE MUTANTS FROM INDIVIDUAL BACTERIA

Information on the kinetics of production of phage material has been obtained by analysis of the distribution of mutants (r and w types) in single bursts of phage T2.

On the one hand, if each new replica of a genetic determinant were produced independently of others produced in the same cell each mutation would give rise to one mutant particle per burst, which would lead to a nonclonal distribution of mutants in individual bursts. On the other hand, if each determinant, after being produced, acts itself as a model for new replicas, the mutants will be present in clones, each clone representing the offspring of one mutation; smaller clones will be more frequent than larger ones, since they stem from later mutations, more frequent because of the presence of a larger population.

Analysis of 3600 bursts gave 67 bursts containing at least one r or w mutant. The distribution is clearly clonal, with clones of mutants ranging in size from 1 to over 40 individuals. The observed distribution agrees with the one calculated, assuming constant probability of mutation per reduplication. The quantitative analysis is not yet complete. The uni-mutational origin of each clone was confirmed by crosses of four pairs of r mutants, each pair from one clone, between members of each pair and among different pairs. All intrapair crosses gave no r^+ recombinants, all interpair crosses gave several r^+ recombinants, proving the allelism (and probable identity) of members of a clone and the nonallelism of members of different clones.

The clonal distribution of spontaneous mutants is the more remarkable since in recombination experiments the recombinants of any one type are distributed nonclonally (Poisson distribution, Hershey and Rotman, 1949, and Doermann unpublished). The combined data indicate the existence of a phase of autocatalytic replication of genetic material during which genetic recombinations have not yet taken place. It confirms the conclusion that little or no reproduction can occur after recombination. In this respect recombination is a terminal phenomenon. However, in order to explain the lack of correlation between opposite recombinant types one has to assume that recombination precedes, or is concurrent with, the formation of fully active particles (cf. p. 11).

MEASUREMENT OF CONCENTRATION OF PLANT VIRUS SUSPENSIONS

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Rational methods for measuring the concentration of plant viruses were initiated in 1927 by McKinney. McKinney used tobacco mosaic virus, and single tobacco plants, as units for infection. Two years later Holmes suggested the use of local lesions for measuring virus concentration. Local lesions are visible injuries caused by virus particles entering an inoculated leaf, infecting it, and multiplying around the points of entry. They have become the principal unit for measuring virus concentration. The use of local lesions is limited to virus-host combinations, which easily produce countable lesions after mechanical inoculation.

A sample of virus, suspended in plant juice, water or some other watery medium, is rubbed lightly over the upper surfaces of a series of leaves. The virus enters through minute wounds into leaf hairs or other surface cells. Entry is almost instantaneous. Where infections occur, viruses multiply locally, spreading to a group of adjacent cells, killing or injuring them. The boundaries of such lesions remain well defined, and the lesions are generally separated by healthy cells from other lesions. Within limits, the more infectious the virus particles in the suspension used for inoculation, the more lesions are likely to appear on the host plant. Different samples of virus may be compared on similar series of leaves, and rated for relative concentration according to the greater or smaller numbers of lesions produced.

The nature of the inoculated surface sharply separates the local lesion method for measuring the concentration of plant viruses from the egg-membrane method for animal viruses. Epidermal cells of the leaf surface are mature, differentiated, and variable. They are protected by a cuticle and a cell wall of cellulose pectin and suberin. No virus placed on this surface can penetrate it directly. Wounding is therefore a prerequisite for entry by the virus and for the initiation of each single lesion. During inoculation, it seems that only a fraction of a percent of the cells on an inoculated leaf surface are wounded in the right way and to the right degree for the entry and establishment of virus in living leaf cells. Making suitable wounds for entry of the virus probably implies either (1) rupturing the cuticle and rigid wall of epidermal cells without destroying the underlying cell protoplasm, or (2) getting virus into injured cells, the contents of which are then

withdrawn through the protoplasmic bridges into neighboring uninjured cells.

At the same time as the wounds are made, moieties of the inoculated virus suspension must make contact with them. The number of contacts will depend on the wetability of the plant cuticle and the spreading power of the applied inoculum. When inoculum is rubbed over a leaf surface during the process of inoculation, there is not complete contact between the film of liquid and the leaf surface. The number of suitable entry points made by rubbing the leaf may be greater than the number with which the inoculum actually makes contact. Evidence of this is obtainable by adding to the inoculum soluble or insoluble substances that improve its wetting power without otherwise affecting the infection process. As the wetting power of the inoculum increases, the numbers of lesions rise, presumably because the inocolum makes contact with more suitable points of entry into the epidermal cells. If equal amounts of the inert wetting agent are added to inoculum diluted to different virus concentrations, the rise in numbers of lesions is proportionately the same at all dilutions.

Presumably, once contact is made between virus suspension and the protoplast of a wounded cell, the physiological condition of the plant will affect (a) the degree of entry of virus particles into cells, (b) the chances of a virus particle reaching those regions of the cell or attaching itself to those molecular configurations in the host protoplasm, which allow virus multiplication to occur. Experiments on the effects of light on host plants before and after inoculation indicate significant alterations in the reaction of host plants to infection. These and other experiments also indicate that, of the many virus particles in the moieties of inoculum that make contact with the naked protoplasts of host cells, only a small proportion infect and multiply.

There is an obvious analogy between the initiation by viruses of local lesions on leaves of susceptible host plants, and the development of bacterial colonies on solid media. This analogy led Youden, Beale and Guthrie, and Bald to formulate the relation between relative concentration of virus and numbers of lesions, on the assumption that numbers of lesions would conform to the term of a Poisson distribution. Some samples of virus when tested by dilution, inoculation, and lesion counts conformed very well. Some did not. To cover cases of divergence, Bald derived a second equation on the assumption that at higher concentrations virus particles may be more or less aggregated, and that single particles and aggregates of more than one particle are

equally effective in penetrating host tissues. Aggregation was assumed to be reversible; as a concentrated virus suspension is diluted, the aggregates break apart. Within the limits of the experimental error the modified equation fitted many dilution series not fitted by the simple equation. An independent summary and discussion of these formulations and the ideas on which they were based is given by Lauffer and Price.

The simple equation for the relations between relative virus concentration and numbers of lesions is:

$$y = N(1 - e^{-pnx}) \quad (1)$$

where y is the number of lesions, and N the maximum number of lesions that can be produced by increasing the concentration of virus particles in the inoculum. This maximum is equal to the number of entry points, or infectable areas, created by inoculation on the inoculated leaves, with which the inoculum effectively makes contact. Its value is supposed to be the same whether or not virus gains access and causes infection through all N entry points. The value, e, is the base of natural logarithms, and the composite value pn represents the mean number of virus particles from a sample of undiluted inoculum entering and causing infection at a single one of the N entry points. The value x is the relative concentration, the inverse of the dilution.

The postulates on which this formulation rests are (1) a single virus particle can cause infection, and (2) the sites where the final acts of infection occur are all equally susceptible. For agreement with the Poisson distribution within the usual limits of experimental error the second postulate need not be strictly true. The following hypothetical series are obtained on the assumption that three types of discrete infectable areas exist in equal numbers on a series of leaves (see Table I).

The numbers of virus particles needed to cause single infections within these three types of susceptible areas are in the ratio 1: 2: 4. Series 1 is derived from numbers of lesions produced by inoculation with a series of dilutions of one virus sample on the leaves bearing these three types of infectable areas. They are represented as percentages of the maximum possible number of lesions (N). A perfect fit with the Poisson distribution for each type of infectable site is assumed. Series 1 is the mean of three such series with values of pn, 10.24, 20.48, and 40.96. It departs slightly but definitely from series 2, having the same value of N and a mean (pn) equal to the average of the mean for the three series (23.89). Suppose, however, that the numbers of lesions from which the values in series 1 were derived had been subject to random variation as in series 3. Series 3 was

1 Mean of 3 Poisson series	2 Calculated series	3 Series 1 subject to random variation	Approximate difference per cent between 1 and 3	4 Calculated series
100	100	98	—2	98
99.8	100	104	+5	98
97.2	99.8	94	—3	97.7
87.9	95.0	87	—1	92.5
70.6	77.7	75	+6	74.9
48.9	52.7	50	+3	50.3
29.8	31.2	29	—4	29.6
16.6	17.0	18	+7	16.2
8.8	8.9	8	—9	8.4
4.5	4.5	5	+2	4.3
N=100 pn= 10.24 20.48 40.96	N=100 pn= 23.89			N=98 pn=23.04

Table I

obtained by adding or subtracting to the terms of series 1 randomly chosen percentage variations of the magnitude shown beside series 3 and rounding off the terms to the nearest whole numbers. In fitting series 3, the value of N was assumed to be 98 instead of 100 and the value of pn at the highest concentration of virus was assumed to be 23.04 (series 4), instead of 23.89 as in series 2. The fit obtained would be considered very good if 3 were an experimental series.

The deviation from a Poisson distribution in series 1 is trivial compared with deviations in numbers of local lesions found in dilution trials with many samples of tobacco mosaic and attributed to aggregation of virus particles. No amount of adjustment will give Poisson series in agreement with the numbers of local lesions found in such trials. The hypothesis of virus aggregation was applied to these cases. The equation formulated to cover them is:

$$y = N(1 - e^{-pvx}) \quad \ldots\ldots\ldots\ldots\ldots\ldots (2)$$

where *pv* is the mean number of aggregates consisting of one or more virus particles that enters to cause infection at a single one of the *N* points. The relation between the number of aggregates causing infection and the number of component infectious virus particles is given by the equation

$$K(pvx)^2 + pvx - pnx = 0 \quad \ldots\ldots\ldots\ldots\ldots\ldots (3)$$

i.e.

$$pvx = \frac{\sqrt{1 + 4K.pnx} - 1}{2K}$$

The value *K* gives an estimate of aggregation. Methods of evaluating the maximum number of lesions, *N*, the aggregation factor, *K*, and the concentration factor *pn* are best outlined by Lauffer and Price.

The postulates on which this formulation is based are the same as for equation 1, except that aggregates of one or more than one virus particle are assumed to have the same chance of entering the plant cells to cause infection. As in the simpler case, however, it is assumed that a single virus particle is capable of causing infection.

These equations are applicable to the end-to-end aggregation of virus particles, or to any other form of linkage involving only two faces on a virus particle. It has not been proved that an equation of this form may be applied, for example, to aggregation of virus particles in a three dimensional lattice. The equation may not be applicable to such viruses as bushy stunt which crystallise in this way. If equation 2 is applied to a virus which aggregates in an unknown or in a different manner, *K* should be considered rather as a measure of the distortion of dilution series than as a measure of aggregation.

This was the point of view adopted even for tobacco mosaic virus before there was good evidence of end-to-end aggregation. However, recent work on the viscosity and sedimentation rates of virus suspensions (e.g. by Schachman) provides independent testimony in favor of end-to-end association of tobacco mosaic particles in moderately dilute suspensions and their dissociation on further dilution. Such physical studies on tobacco mosaic virus have provided evidence in support of the original postulate: reversible aggregation of the virus particles does contribute to distortions of the lesion-concentration relationship.

An alternative explanation for such distortion has recently been suggested by Kleczkowski, who has attempted to fit the results of lesion-virus concentration trials by a single hypothesis. Kleczkowski suggested that the infectable areas on leaves exposed by inoculation with a virus suspension differ in infectability. The chance of infection is considered in terms of the numbers of virus particles that must make contact with

each infectable area to cause an infection. The infectability of these areas is assumed to follow a normal frequency distribution.

Kleczkowski's hypothesis assumes that the type of distortion of the lesion - dilution relationship illustrated in Table I may become much greater because of the greater variability of the infectable areas on the inoculated leaves. Such an explanation does not easily account for the common experience that dilutions from different samples of the same virus inoculated on similar sets of plants may give dissimilar lesion-dilution curves. If Kleczkowski's hypothesis were correct, differences in the form of dilution curves obtained in parallel trials on similar sets of plants should be relatively slight.

Kleczkowski has shown in experiments with purified samples of virus at concentrations between 1 and 20 gms. per litre, that numbers of lesions increase with virus concentration far above the level at which the maximum, N, would usually be supposed to lie. He therefore discounts any fit dependent on a value of N that would give agreement with a Poisson series or with a Poisson series modified for aggregation.

Details are given by Kleczkowski of 4 experiments with purified tobacco mosaic virus in which concentrations of 1 to 20 gms. per litre were tested as well as lesser concentrations. The results of these 4 experiments are plotted in Figure 1 as log local lesions against log concentration in grams per litre. The scales for log lesions in each experiment were adjusted on the ordinate so that the values for the higher concentrations fell on a single curve relating lesions and concentration.

The results presented in this way indicate that the 4 samples of tobacco mosaic virus induced a similar progressive increase in relative number of local lesions with increasing concentrations of virus between 0.1 and 20 gms. per litre. Coincidence at these concentrations of the curves from the different experiments suggests a common mechanism for the relationship between lesions and concentration. This mechanism is presumably not the same as that inducing dissimilar curves with more dilute virus suspensions. Amounts of 0.1 to 20 gms. of tobacco mosac virus, like somewhat smaller quantities of certain inert materials (see above), alter the physical properties of the inoculum. The virus itself acts as the spreading or wetting agent in the inoculum. The curve in the upper ranges of concentration presumably demonstrates the rise in value of N as the wetting power of the inoculum increases. Virus is enabled to make contact with more and more infectable areas on the leaf surface as virus concentration is raised.

Values of N for inocula at lower concentrations may be derived

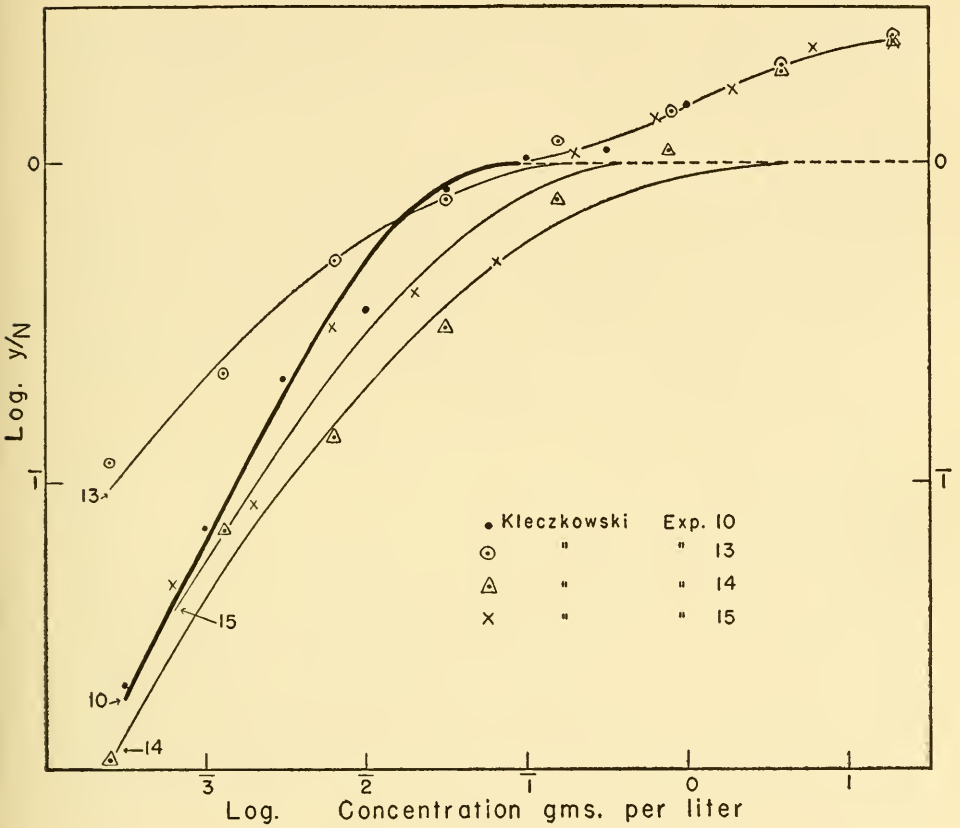

Fig. 1. Data from 4 experiments by Kleczkowski on relationship between virus concentration in inoculum and numbers of local lesions produced on inoculated test plants. Plotted as log numbers of lesions (y) over estimated maximum number of lesions (N) produced by inocula assumed to have constant physical characteristics. Points above the value log y/N=0 are interpreted to indicate a uniform proportional increase of lesions in all 4 experiments. This is due to alteration in the physical properties of the inocula produced by increasing quantities of virus over 0.1 gm. per litre. Curves below the value y/N=0 for experiments 10, 13, and 14 were fitted according to equations given in the text; the curve for experiment 15 was not fitted because of the variability of the data, but was given approximately the form produced by a lesser degree of aggregation than 14.

from the horizontal dotted line in Figure 1. They are for experiment 10, 2500; experiment 13, 1250; experiment 14, 590; and experiment 15, 350. With these as maxima the more dilute portions of these curves may be fitted, for experiment 10 on the assumption that no aggregation of virus particles occurred, and for the other three on the assumption of aggregation. Experiments 13 and 14 may be fitted with the same value of K but with different values of pv. The results obtained in this way imply that in these two experiments the virus in the inoculum was aggregated in the same degree, but the infectable areas on the leaves of the test plants in experiment 13 were twelve times as susceptible as those in experiment 14. On the other hand, although in experiment 10 there was no aggregation and in experiments 14 and 15 the degrees of aggregation were different, the infectable areas on the leaves of the test plants were about equally susceptible.

For the most part in these four experiments no effects of the virus on the physical properties of the inoculum need be invoked to explain departures from the Poisson relationship at the lower virus concentrations, i.e., at levels below the horizontal hatched line in Figure 1. Above this line is a point representing a single virus dilution in experiment 14 that is displaced to a position below the upper curve. This may be interpreted as the one obvious example of interaction between the physical effects of virus in the inoculum and the effects of infectivity below the maximum needed to present active virus to all infectable areas with which contact is made on the leaf surface. Otherwise, these experiments may be interpreted to indicate only slight interaction between the physical effects of increasing virus in the inoculum and effects arising from the infective capacity of the inoculum.

These experiments from Kleczkowski's paper have been discussed at some length to show that the original simple explanation of lesion-virus concentration curves based on the Poisson series may still be proved reasonably adequate. Some variability in the susceptibility of infectable areas is not denied, but it is suggested that this is not the main cause of the heterogeneity of lesion-concentration curves. For the tobacco mosaic group of viruses and probably for others aggregation of virus particles will explain much of this heterogeneity. It is becoming possible at will to cause aggregation or dispersion of virus in suspensions of tobacco mosaic virus and to estimate aggregation by various physical methods. Thus independent experimental evidence of the effects of aggregation on the lesion-virus concentration relationship may be obtained.

Another postulate which has been used to explain the relationship

between numbers of infections and virus concentration is that an infection cannot be caused by a single virus particle, but that more than one is needed to cause each infection. Lauffer and Price have shown that lesion-virus concentration curves cannot be accurately fitted on this assumption.

Equation No. 1 above is essentially the same as the equation that has been used to describe the relation between concentration of an animal virus and the number of lesions on inoculated egg membranes, with this difference. Animal viruses may cause infections at extreme dilution, where numbers of lesions are approximately proportional to concentration. Plant viruses produce countable numbers of lesions only at much higher concentrations because so few virus particles of those applied enter the host plant and cause infection. Plant virus concentration and numbers of lesions are generally not proportional. The part of the dilution curve over which measurements are often taken has a changing slope. The relationship between numbers of lesions and concentration of unknown samples of a virus that one may wish to compare cannot then be predicted. There is the additional possibility of complications due to aggregation of virus particles, which adds to the uncertainty of deductions from lesion counts that one virus sample is more concentrated than another. Strictly speaking, it has seldom been possible to assume without further evidence that one sample of virus is more concentrated than another because it produces more local lesions. In practice this assumption is often made, and may often be correct. If there is a wide difference in numbers of local lesions, its correctness is very probable.

This uncertainty rests on the difficulty of obtaining an estimate of N, the maximum possible number of lesions, *i.e.*, the number of entry points or susceptible regions on the inoculated leaves. If N can be determined and is the same for both samples it seems reasonable to assume that the sample of virus producing more lesions contains more infectious units. However, if the virus under test is subject to aggregation of its suspended particles, the infectious units in the two samples may represent different degrees of aggregation, and the sample producing more lesions may not contain the greater number of infectious virus particles. Obtaining a true estimate of relative virus concentration in aggregated samples would involve the solution of equation 3 above for each sample. To make direct comparison of two samples on the basis of lesion production, the constant K, which indicates the degree of concentration, should be zero (no aggregation) or have approximately the same value for the different samples.

The equality of the two constants N and K is the basis for a direct

comparison of virus concentration on the basis of lesion counts. If the values of N and K as well as values for pv or pn can be estimated from the data, it is easy to calculate the relative numbers of virus particles in the tested sample. However, the absolute numbers of virus particles are still not calculable. Although it may be possible to estimate the volume of inoculum applied to a leaf surface, it is not possible to decide how much of it makes contact with the scattered susceptible regions of the leaf produced by wounding during inoculation. That is, the amount of inoculum applied effectively is unknown. It is further impossible to estimate the proportion of the unknown number of virus particles in this amount of inoculum making contact with susceptible regions of a leaf, that enters and causes infection. There is at present no way of estimating absolutely the number of infectious virus particles in a sample of a plant virus. It is possible to discover by physical means the number of standard-size particles in a sample, say of a tobacco mosaic inoculum, but this is not necessarily the same as the number of infectious virus particles.

Price and Spencer, in a series of four papers, attempted with some success to by-pass the necessity for calculating values for N and K. They conducted local lesion trials with two or more dilutions of each virus sample. This allowed them to insert a factor for slope in the determination of the effect of dilution on the numbers of lesions produced. Slope was a term for the regression of lesions (log values) on concentration and it automatically corrected the estimate of difference in the ratio of lesion counts between two virus samples.

The discovery in recent years of methods for keeping suspended virus particles dispersed rather than aggregated, offers a chance largely to eliminate aggregation as a source of error in estimating virus concentration. This being so, it becomes more feasible to make use of the range of virus concentration where lesions and concentration are proportional. The addition of inert substances to the virus inoculum to increase contact between the inoculum and the surface of inoculated leaves will increase the number of lesions and favor the use of dilute virus suspensions. Celite has recently been used by several workers for this purpose (personal communication from Dr. E. M. Hutton). The effect of fine carborundum powder in the inoculum, which also aids in wounding leaves with a tough cuticle or smooth hairless surface, may be partly of this kind. Raising the susceptibility of test plants by cultural treatment or submitting them to reduced light for some hours before inoculation may also increase the numbers of lesions. Such simple modifications of technique may eventually enable workers to use virus preparations at dilutions high enough to be within the range

where numbers of lesions are proportional to virus concentration. This appears the simplest method of obtaining direct estimates of the relative concentration of infective virus particles. For such low virus concentrations there are established statistical procedures for estimating concentrations and experimental error. These have been used in association with the egg membrane technique for the bioassay of animal viruses. At present for the reduction of local lesion data and the estimate of experimental errors the most reliable method, introduced by Kleczkowski, involves preliminary conversion of values to log lesions plus a constant. The constant is estimated in each instance from the local lesion counts themselves.

A serious cause of variability in the local lesion method is the great range of susceptibility to infection between plants and between leaves on the same plant. This variability is mainly physiological; it is found amongst plants from carefully selected clones as well as amongst plants grown from seed. Methods of designing experiments similar to those used in agricultural field trials overcome much of this variability. The half-leaf method first suggested by Samuel and Bald has been widely used to reduce the variability of lesion counts. Samples of virus to be compared are inoculated on the opposite halves of the same leaves. Comparisons of each sample may be made with a standard sample of virus, or samples may be paired in all possible combinations for inoculation on opposite half leaves.

The limits of accuracy attainable by the local lesion method vary widely according to the details of experimental arrangement, technique, environment, virus, and host plant. It is possible under favorable conditions to prove the significance of differences between lesion counts as small as 10 per cent. Where host tissues are variable and techniques inefficient, differences of 50 per cent may not be significant. A range between 15 and 25 per cent is a fair estimate of the extent of differences necessary for significance in careful and accurate work. In the series of papers by Spencer and Price already cited, significant differences were generally found at this level.

In the ordinary use of the local lesion method many of its theoretical shortcomings are ignored. In effect, competent workers adopt measures to reduce the chances of variation in the constants N and K, although they don't generally describe their precautions in that fashion. Virus samples as far as possible are suspended in uniform media and inoculated on uniform test plants in a uniform way. When care is taken to eliminate variables it seems, in practice, that comparisons of lesion counts give reasonable estimates of relative virus concentration. It is seldom, however, that any real attempt is made to give these esti-

mates in terms of proportionality. Sometimes one sample is said to be, say, twice as strong as another: what is meant is that one sample has produced twice as many lesions as the other. This is obviously not the same as having twice the number of infectious virus particles per unit volume of inoculum.

The possibilities of using other units than local lesions for estimating the concentration of plant viruses have been somewhat neglected. Many viruses readily infect inoculated plants but do not produce local lesions. Others produce diffuse lesions evident on inoculated leaves, but not discrete and countable. It is thus possible to use whole plants or single leaves as the infection units, and the value of N in equation 1 (or 2) is then known. The plants used for such inoculation tests need to be very uniform, as there are some practical and theoretical objections to the comparison of infection counts made with variable units such as these may be. However, the Poisson distribution is not very sensitive to distortion from heterogeneity of the units entering into the estimate of the mean (pn or pv). Some of the theoretical objections do not seriously interfere with the use of leaves and plants as units for infection, provided the usual precautions necessary in quantitative work are taken. One serious disadvantage is the amount of material and space needed for trials, particularly with whole plants as units.

The adaptibility of infection methods for measuring the concentration of plant viruses is shown by the following example taken from a paper by Bennett on sugar beet curly top virus. Insect vectors of curly top were fed on three lots of partially purified curly-top virus. One sample of virus was the exudate from vascular tissues (phloem) of diseased beet plants, one from expressed juice of beet plants, and one from ground up bodies of insect vectors carrying the virus. Batches of noninfectious vectors were fed through membranes on serial dilutions of each of the three virus samples sweetened with sugar. The insects were then placed singly on healthy test plants. In each instance about 42 per cent of the insects appeared to be the maximum number causing infection on the test plants under the conditions of the experiment. N, the maximum number of plants infected in each trial, was taken at 42 per cent of the number inoculated from each dilution by single insects. The numbers of infected plants were fitted in two instances by the simple equation (1) on the hypothesis there was no aggregation in the original virus suspensions fed to the originally noninfectious insects. The third (the phloem exudate) appeared to be aggregated, as it was fitted by the more complex equation. The results of fitting these dilution series are shown in Table II.

Dilution	Phloem exudate		Whole beet juice		Ground-up infective insects	
	Observed	Calculated	Observed	Calculated	Observed	Calculated
1:1			41.5	42		
1:10	76	72.1	25.5	29.3	35.5	41.7
1:20	60	65.3				
1:50	53	51.3	12.0	8.9	27.5	28.0
1:100	37	39.0	7.0	4.7	13.5	17.7
1:200			2.0	2.4	15.5	10.1
1:300			0.5	1.6	6	7.1
1:500	18	15.2			6	4.4
1:1000	9	9.0			1	2.2

Sample	Relative Concentration	Condition
Phloem exudate	150 (33)	Aggregation (K = 1.58)
Beet juice as a whole	4.5 (1)	No aggregation
Leaf hoppers	55 (12)	No aggregation

Table II

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INTERFERENCE PHENOMENA WITH PLANT AND BACTERIAL VIRUSES

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In work with plant viruses, the ability of one virus to protect plants against another is widely used to identify clinically different viruses as related strains. Almost all serologically related viruses that have been studied have been found to be mutually antagonistic when present together in plants, and a plant systematically infected with one strain fails to develop symptoms characteristic of another serologically related strain when reinoculated with it. The protection is not always complete; in time a second strain may predominate over the first if it is one that is more invasive or multiplies more rapidly. Although there is no reason to connect the phenomenon with antibody production in plants, there is evidence that the degree of protection afforded by one strain against infection by another is correlated with the number of antigens they have in common. One exception to the rule that serologically related viruses are mutually antagonistic is provided by potato virus Y and tobacco veinal necrosis virus. Although they share common antigens, tobacco plants systematically infected with one succumb to the other when inoculated with it.

The plant protection phenomenon superficially resembles the mutual exclusion phenomena described with bacterial viruses, but there is one striking difference. Mutual exclusion occurs with bacterial viruses that are *not* serologically related, such as T1 and T2, whereas serologically related strains of one bacteriophage, e.g., T2 and T2r, can both infect the same cell, multiplying in it, and such multiple infections give new strains that combine characters of each parent type. Such related strains are considered not to interfere with one another, but this would seem a terminology that does not accurately reflect the course of events in mixed infections and one that has been used in contrast to the seemingly greater interference between viruses that show the phenomena of mutual exclusion. The differences between the behaviour of serologically related strains of plant and bacterial viruses are probably less than appear at first sight and they mainly reflect the different terminologies used in the two subjects.

If two related strains of a bacterial virus enter a bacterium, both multiply, but the final yield of virus is not greater than would have occurred from infection with one strain alone. Also, if a large excess of one strain enters, the second may be undetectable. This is precisely similar to the results obtained when plants are simultaneously infected with two strains of one virus. Both will enter and multiply, and the final yield of total virus will about equal that produced by either strain

alone. Symptoms will be intermediate between those caused by the two strains separately and the yield of each strain, and the symptoms, will reflect the relative proportions, of the two strains in the inoculum. It has not been established that the two strains multiply together in individual cells, but there is no reason to assume that they cannot. The protection of a plant by one strain against another is a later phenomenon, produced when one strain has previously multiplied to a high virus content. It seems there is a maximum amount of any one virus that a cell can produce, and if it has already produced this amount with one strain another serologically related one cannot become established and multiply. This phenomenon is one for which tests cannot be made with bacterial viruses, because when one strain has multiplied fully, lysis occurs. There is nothing comparable to a systematically infected plant with bacteria, and the apparent differences beween the behaviour of strains of plant viruses, on the one hand, and of bacteriophages, on the other, probably reflect differences in host behaviour. Strains of both kinds of viruses probably interfere with the multiplication of one another to a comparable degree.

Despite the seeming similarity between the plant protection phenomenon and that of mutual exclusion, it is in mixed infections with unrelated viruses that plant viruses and bacteriophages show greatest differences. There is nothing known with plant viruses that is exactly comparable with the mutual exclusion phenomenon. Various kinds of interactions are known between different plant viruses, but there are no pairs known in which infection with one precludes infection with another. The nearest approach is provided by tobacco severe etch virus and potato virus Y; plants infected with the first do not support the multiplication of the second, but the effect is not reciprocal and plants infected with virus Y are as susceptible as uninfected plants to severe etch virus. As severe etch virus multiplies, virus Y decreases and the etch virus can replace and supplant virus Y in tissues where it was already fully established.

Mixed infections with pairs of unrelated viruses are common with plants, but only a few have been studied quantitatively. Often, though not always, such mixed infections produce symptoms that are more severe and of a different type than those produced by either virus acting alone. With some pairs, both viruses reach as high a concentration as they would if present alone; with other pairs, one or other virus may be reduced in amount, whereas in mixed infections of severe etch and dodder latent mosaic virus, the concentration of the dodder virus may be greater than in comparable plants infected with it alone. It is likely that in mixed infections the different viruses occur together in the

same cells, but only with tobacco mosaic and severe etch viruses is there positive evidence for this. In plants simultaneously infected with these two viruses, intracellular inclusion bodies characteristic of each virus regularly occur in the same cells.

In summary, it can be said that related strains of bacterial viruses probably interfere with one another in the same manner as do related strains of plant viruses, but the lysis of infected bacteria prevents the testing for any phenomenon comparable to the protection afforded to plants systematically infected with one strain against the effects of a second. Serologically unrelated plant viruses interact with one another in a variety of different ways, but there is nothing similar to the mutual exclusion phenomena found with unrelated bacteriophage. This again may reflect host differences rather than differences between the viruses that attack the two kinds of host. The bacterial cell is obviously more drastically affected by infection than cells of higher plants in which systemic infection occurs. The early effects of infection with one bacteriophage probably injure the host metabolism too severely for another bacteriophage to be able to multiply.

COMMENT

M. DELBRÜCK

The foregoing discussion by Mr. Bawden explains satisfactorily the apparent discrepancy between the behaviour in mixed infections of pairs of *related* plant viruses on one hand and bacterial viruses on the other. In both cases interference occurs of a type which is best explained in terms of competition for intracellular material available in limited amount. In *simultaneous* infections with related strains of viruses the two viruses prorate the available material and give correspondingly reduced crops.

At first sight this looks like a competition for something that might be termed a "precursor" of virus. However, such an inference would be weakly founded. In the first place, we do not know whether this material is of the nature of *building material* for the virus or of the nature of *equipment* necessary for the synthesis of the virus. In the second place, if it is a question of building material, we do not know how large a part of the virus is involved; perhaps it is only a matter of one or another small molecular group in the virus. In the third place, we do not know whether the material is specific, i.e., whether the material for which one group of related viruses compete is or is not the same as that for which another group of related viruses compete. In

bacterial viruses our inability to settle this point is due to the phenomenon of mutual exclusion: unrelated viruses simply do not multiply within the same cell, therefore we can not tell whether, if they did multiply within the same cell, they would compete for a certain material. In plant viruses it should be possible in principle to settle this point but it does not seem to me that it has in fact been settled satisfactorily. In the preceding discussion a number of cases are cited in which mixed infection with unrelated strains apparently gave no mutual exclusion. Such experiments are not decisive for deciding whether or not mutual exclusion occurs. They correspond to mixed infections of *whole bacterial cultures* with two unrelated viruses. From such experiments mutual exclusion could never have been inferred, since in whole cultures some cells will yield one virus, some the other, thus obscuring the mutual exclusion phenomenon which concerns the individual cell. The case of TMV and severe etch, cited by Bawden, where inclusion bodies corresponding to both viruses are regularly seen in the same cell, is convincing, but not sufficient to prove the rule. We should be prepared to find that some pairs of unrelated viruses parasitize different subcellular structures, and therefore do not exclude each other. Dr. Bald stressed the contrast between the local lesion technique in plant as opposed to animal viruses. In animal virus assay on egg membranes we deal with a continuous layer of infectable cells, while in plant virus assays on leaves only a very limited number of leaf cells may be made directly infectable through injury. Dr. Bald pointed out that this may be the reason for most of the unsatisfactory features inherent in the method of local lesion counts. It seems to me that this defect implies also an advantage which may not have been sufficiently appreciated. If it is true that the primarily infectable cells with which the inoculum makes contact are few and widely separated, or, if inoculation methods can be specifically designed to produce widely spaced lesions, then this method affords an ideal set-up for the study in *individual cells* of mutual exclusion, and of other phenomena of mixed infection, like multiplicity reactivation and recombination. The leaf surface may be smeared with a mixture of the two viruses in relatively high concentration so that every infectable cell is actually exposed to both viruses. At the same time one can be certain that only very few cells, well isolated from each other, will be infected. The first generation of mixedly infected (or, at least, mixedly exposed cells) is thus automatically implanted in host tissue suitable for cultivation of the crop of viruses from these primarily affected cells. The analysis of the resulting local lesions would correspond to the analysis of plaque contents as used by Hershey and by us in earlier studies of recombination; perhaps it may not even be entirely out of the question to perform

direct analyses of the contents of the primarily injured and infected cells, a few hours after inoculation, when the first symptoms of infection become microscopically identifiable. This would correspond to the single burst technique of analysis of mixed infection phenomena, which has been the mainstay of the detailed analyses in phage.

The principal purpose of these remarks is to urge the plant pathologists to pursue mixed infection studies on the *cellular level*, an objective which has proved fruitful in phage beyond any expectation.

THE VARIETIES OF MACROMOLECULES IN EXTRACTS FROM VIRUS-INFECTED PLANTS

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Purified preparations of some plant viruses, for example, tomato bushy stunt, show no signs of heterogeneity when subjected to customary physical tests, and the properties and gross chemical constitutions of preparations made at different times are also reproducible. Despite this seeming appearance of uniformity, there is no reason to consider that the preparations are homogeneous in the sense that every particle has precisely the same chemical constitution and biological activity. There is no method whereby such a uniformity could be demonstrated and there are many ways in which particles could differ from one another without any differences being detectable. The probability that infective and non-infective particles could occur but be undetected is obvious from the fact that inactivation by various methods does not change the morphology of the particles or their gross chemical, physical and serological properties.

To produce a single infection with any of the plant virus preparations so far studied, inocula must contain many thousands of particles. This can be explained away by postulating that most of the particles are wasted because they never encounter a susceptible site on the rubbed leaves or that infection occurs only when a large number of identical particles enter a cell simultaneously, but it is also possible that even the most infective preparations do not consist exclusively of infective particles. The local-lesion assay method allows accurate comparisons of the relative infectivities of different preparations of a virus, but there is no method of estimating the proportion of the particles in any one preparation that is infective. Physical tests for detecting inhomogeneity also are too insensitive for positive conclusions about the uniformity of particles in purified virus preparations. They will fail to distinguish between particles that differ in mass by 1 or 2%, and it is worth stressing that 1% of the particle of even a small virus weighs as much as a haemoglobin molecule. This limitation in the precision of our physical methods, a limitation that is likely to persist for many years to come, means that only heterogeneity can be demonstrated, not homogeneity.

There is now ample evidence that extracts of plants infected with certain viruses do contain a mixture of related but different substances, which are either specific to infected plants or occur in healthy plants in much smaller quantities. Although many claims have been made about the homogeneity of preparations of tobacco mosaic virus, this is one with which such mixtures are readily demonstrated. By following

the usual methods of isolation, preparations of a specific nucleoprotein can be made with fairly consistent properties, although sedimentation boundaries are usually diffuse and electron microscopy always shows particles with different lengths. More critical methods of fractionation, however, show that the nucleoprotein in the original extracts is heterogeneous and occurs in particles with widely different properties. If the extracts are subjected to alternate cycles of centrifugation at 30,000 and 80,000 g., a series of fractions is separated containing particles with different average lengths and consequently with different physical properties and serological behaviour (Bawden & Pirie, 1945b). The infectivity per unit weight of nucleoprotein in such fractions also is correlated with the mean particle length, the most slowly sedimenting material being almost non-infective and the most rapidly sedimenting being most infective. The particles in all the fractions can aggregate linearly to give forms that sediment more rapidly and show intense anisotropy of flow. After such changes all the fractions have similar general chemical, physical and serological properties, but they maintain their original differences of infectivity. Aggregating the small particles does not increase the infectivity; length *per se*, therefore, is no guarantee of infectivity and it is obvious that particles cannot be assumed to be infective merely because they achieve a certain length.

The origin and significance of these various kinds of particles is uncertain, but there are various possible interpretations. The simplest is to assume that there is one unique particle capable of initiating infection, that this duplicates itself exactly, and that all variants arise by degenerative changes in this particle, produced *in vivo* or *in vitro*. There are many treatments that can destroy infectivity without destroying serological specificity, although *in vitro* the only one known to break large particles of tobacco mosaic virus into small serologically active fragments is ultrasonic irradiation (Oster 1947), and under most conditions linear aggregation seems more likely to occur than fragmentation.

There is no *a priori* reason to assume that the ability to cause infection necessitates a unique structure or that infection must necessarily alter protein metabolism so that only one specific product is formed. Indeed, the frequent production of clinically distinct mutants shows that all the particles produced during virus multiplication are not identical. To be infective, particles presumably will need a minimum number of specific groups, but some parts of the particles could probably vary without destroying infectivity. Indeed this is suggested by the fact that most of the amino groups and some of the phenol and indole groups can be substituted without decreasing infectivity

(Schramm & Müller 1940: Miller and Stanley 1942), although an alternative explanation of this may be that the substitutions are reversed by the host cells before infection occurs.

During the course of infection, too, it is likely that a range of similar but not identical substances is synthesized. Luria has suggested that the synthesis of virus particles is the assembling together of macromolecular substances each of which is synthesized separately in infected cells. If this is so, incomplete or faulty assemblies are sometimes to be expected, and the material with the general characters of the virus but lacking infectivity may be examples of such phenomena. Particles of such material fall short of the full stature of virus particles, but the fact that they are non-infective does not necessarily mean they are devoid of activity in the cells where they were produced. The evidence with irradiated bacteriophages suggests that two particles in which different activities have been destroyed may together initiate infection though neither can alone, and non-infective particles of plant viruses may also have some effects for which as yet we have no method of testing.

That infection leads to the production of more than one kind of anomalous protein is also suggested by work on turnip yellow mosaic virus (Markham & Smith 1949) and the Rothamsted tobacco necrosis virus (Bawden & Pirie 1945 a: 1950). Purified preparations of turnip yellow mosaic virus contain particles all of similar size, which have the same isoelectric point and electrophoretic mobility, contain the same antigens and which crystallize in the same form. Only 80% of the particles, however, seem to be nucleoprotein, the remainder being an apparently similar protein but free from nucleic acid. The ability to initiate infection appears to be restriced to the nucleoprotein, but there is no evidence to show whether all or only a proportion of the particles can do so. Extracts from plants infected with the Rothamsted tobacco necrosis virus also contain at least two kinds of specific particles. Both these appear to be nucleoprotein, one being about twice the diameter of the other. Apparently homogeneous preparations of the smaller particles can be made, but these are, weight for weight, less infective than inhomogeneous preparations containing large particles. Mixed preparations can be fractionated by differential ultracentrifugation and by precipitation at pH 4 in the presence of some normal leaf proteins. The material that sediments more readily and precipitates at pH 4 carries more infectivity than the other, but has less serological activity. The large particles could be aggregates of the smaller ones, though this has not been demonstrated. Nor is it proven that infectivity is a property specific to the large particles, though it is clear that many of the small

ones are incapable of initiating infection. As with tobacco mosaic virus, there is no evidence to show whether the small particles are more likely to be stages in the synthesis or in the degradation of fully active particles.

Preparations of the tobacco necrosis virus made by the ultracentrifugation of recently expressed sap are less infective than those made from duplicate lots of sap allowed to age for two days before centrifugation, although otherwise the properties of the preparations do not greatly differ. This suggests that infectivity is a property acquired by many particles during extraction or after they are extracted from infected cells. The acquisition of infectivity has two equally plausible interpretations. It may come from some terminal process which occurs in the sap and adds an essential grouping or rearranges existing groups. Alternatively, the virus particles may be liberated fully formed into the sap but be combined with some cell constituents that act as inhibitors of infectivity, and removal or destruction of the inhibitors may be responsible for the activation. The second possibility has some interesting consequences. Purified viruses combine to form complexes with a range of substances, some of which are powerful inhibitors of infectivity, and it is reasonable to assume that combination with some specific cell constituents is also an essential step in the initiation of infectivity. The inhibitors presumably act because they combine either with parts of the virus that are concerned with this initial combination or with the specific cell constituents. If virus particles were incompletely dissociated from the cell components in combination with which they are synthesized, inhibition of infectivity could be expected, for there is no more simple or effective method of blocking a lock than to leave a part of the key in it. Studying the extraneous material in poorly infective virus preparations may prove a fruitful source of information about the mechanism whereby viruses are produced.

There is no reason to consider that the three viruses mentioned above are exceptional in causing the production of a range of substances, only some of which are infective. Nor is there any reason to assume that the phenomenon is one peculiar to plant viruses. Work on the complement-fixing antigens that are produced at different times during infection with influenza virus, shows that material with the antigenic specificity of the virus is produced before any infective particles can be detected (Hoyle 1948). Analogies could obviously be drawn between this non-infective material and the non-infective nucleo-proteins that carry the serological specificity of tobacco mosaic virus or with preparations of the Rothamsted tobacco necrosis virus whose infectivity increases as they are exposed to the environment of

plant sap. With which system the influenza virus is more comparable remains to be determined, for there is no evidence to show whether the serologically active material ever develops into infective particles, either by subsequent assembly or by the removal of inhibiting materials.

To many virus workers, the infective particles may seem the only important ones, and it is understandable that most work has so far been directed towards obtaining apparently homogeneous preparations with the highest infectivity. Nevertheless it would be a mistake to focus attention exclusively on the infective particles or to place too much reliance on physico-chemical and serological tests as indicating biological uniformity. Already, it is obviously misleading to assume, as is commonly done, that any anomalous particles which can be sedimented from infective extracts in the ultracentrifuge or resolved by the electron microscope, are necessarily capable of infecting a new host. Many such particles, although specific to infected cells, are non-infective, and whether they represent intermediate stages in the synthesis of infective particles, degradation products, or complexes with host constituents, their further study can hardly fail to illuminate the complex processes that proceed in virus-infected cells.

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INSECT VIRUSES

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Characteristic for one type of virus disease of insect larvæ are polyhedral shaped inclusion bodies. These polyhedra develop in great numbers and very rapidly in the nuclei of most organs, shortly before the death of lepidopterous larvæ (Bergold, 1943) and in the mid-gut nuclei of sawfly larvæ (Bird, 1950). Finally the nucleus and cell membranes burst and enormous numbers of polyhedral bodies are released into the serum.

They can be purified by centrifugation and are finally obtained as a white powder of considerable purity. Their size and crystal shape vary with the insect species. The polyhedral bodies from silkworms are about 5 microns in diameter and are rhombical-dodecahedra.

The polyhedral bodies are insoluble in water, but dissolve rapidly in weak alkali, a condition which exists in the gut of susceptible insect larvæ. Some years ago I was able to demonstrate (Bergold, 1947) that about 95% of the weight of the polyhedral bodies consists of the very homogenous, but not infectious, polyhedral protein, with very little or no nucleic acid and a molecular weight of 275,000 to 375,000, depending on the insect species. Only about 3 to 5% of the polyhedral bodies consist of the active virus particles which are occluded in the polyhedral bodies. These active virus particles are liberated when the polyhedral bodies are dissolved in weak alkali.

The nature of the polyhedron-virus union is unknown. The polyhedral protein and the virus particles from silkworms are serologically unrelated, they have also no relationship to silkworm serum (Bergold and Friedrich-Freksa 1947).

The virus particles are rod-shaped with dimensions of about 30 to 50 millimicrons in diameter and 260 to 360 millimicrons in length, depending on the insect species. About 10^{-13} gm. or about 100-1000 of these rods are necessary for the L.D. 50. Infectivity tests combined with sedimentation experiments in the ultracentrifuge and serological examinations give very strong evidence that these rods are identical with the infective principle (Bergold, 1947, Bergold and Friedrich-Freksa, 1947). The virus rods contain about 14% desoxyribonucleic acid. Acid hydrolysis and paper chromatography (Wellington, 1950) indicate the presence of the following amino acids: cyst(e)ine (small), aspartic,

glutamic, serine, glycine, threonine, alanine, methionine, tyrosine (small), valine, leucine + isoleucine + phenylalanine, proline (small), lysine, arginine, histidine.

The sedimentation curve of virus suspensions, obtained in the ultracentrifuge, are rather wide or show even different fast sedimenting components. This means that the virus particles are not homogeneous in size (Bergold, 1948a).

Extensive observations in different electron microscopes of many different preparations of four different viruses confirmed this assumption, showing different morphological forms of virus sizes. It led finally to the discovery of a development cycle (Bergold, 1950). Different developing forms have been discussed also in other viruses (Hoyle, 1948, Heinmets and Golub, 1948, Bang, 1948, Markham, Matthews and Smith, 1948, Horsfall, 1949). I would like to repeat that the starting material for these insect virus preparations was very well purified crystalline polyhedral bodies, dissolved in weak alkaline. The only possible inclusions must originate in components of nuclear or virus material. Since Trager (1935) has shown in tissue cultures that the polyhedra develop within a few hours, it seems possible that various developing stages of the viruses are occluded and liberated when the polyhedral bodies are dissolved in alkaline. From several hundred pictures a sequence has been selected which was probable from the viewpoint of morphological development. The continuity of morphological characteristics gives strong evidence that we are dealing with the virus.

The early stages, growing from unknown size, are spherical. The "germ" elongates and curves as it develops within an outer spherical membrane. As it grows it straightens out to a more rod shaped particle. While still in the membrane it shrinks in width and the rod finally leaves the spherical and a tube shaped membrane. It can not be decided yet, whether the rods slip out of the tubular membrane due to the high vacuum in the electron microscope or whether it happens under natural conditions. The characteristic breakage of the empty membranes confirms their reality. Without any comments I would like to recall to your mind the "ghosts" of some bacteriophages, which proved to be membranes (Anderson, 1949). The spherical membrane of the insect virus particles confirms the connection between the spherical developing forms and the mature rods. Sometimes a lengthwise groove in a rod is visible. This leads to the question of multiplication.

We do not know how the rod shaped particles begin again to multiply. Two possibilities are suggested. Either the rod shaped particle shrinks to a small sphere or it contains several sub-units. The first pos-

sibility is very unlikely, although a similar process takes place at the sporulation of Clostridium tetani when the rod-like fusion nucleus changes into a small sphere (Bisset, 1950). The second possibility may be supported by the following argument:

If only one rod develops from one, no multiplication takes place at all. In the silkworm virus the double rods occur infrequently and the rapid increase of the virus particles can hardly be explained. It is therefore possible that a single rod contains smaller sub-units which can develop into rods. If virus rods are suspended in 0.05 M. HCl small spherical bodies become visible. This is of course no proof, it may be just an artifact due to denaturation. There is good hope of showing sub-units also in untreated rods applying shadow casting. I have applied several methods to divide the rods into sub-units and check their infectivity, but without success. The existence of sub-units has been suggested in other viruses too (Luria, 1947, Putnam and Kozloff, 1950).

The developing stages of another insect virus, the polyhedral virus of the spruce budworm, are similar to those from the silkworm. Double rods are more frequent here. Bundles or developing stages and mature rods seem to be present at the same time in one polyhedral body.

The development of the polyhedral virus of the gypsy moth is a little different since usually four rods develop in a bundle arrangement. Again the early spherical stages and the curved "germ" within the membrane which seems to undergo heavy internal structural changes. Lengthwise fission is the rule here, but sometimes narrow forms with probably only one rod can be found. Finally usually four mature rods slip out of the membranes. In partly dissolved polyhedral bodies many empty pockets can easily be seen from which the virus bundles have slipped out.

The second type of insect viruses we know of is the capsule virus (Bergold, 1948b). In contrast to the polyhedral virus, the inclusion bodies are egg shaped and much smaller, that is only about 360 x 230 millimicrons. Each capsule contains only one virus rod or a double rod. The rods slip out when the capsule is dissolved in alkali. In spite of these differences, the development of the rods is similar to the polyhedral virus. Again the early spherical stages, the straightening out of the "germ" inside the membrane, and the double forms. The mature rods are somewhat different: wider and usually slightly bent. In such capsule virus suspension I have never observed tube shaped membranes.

The "life cycle" and the complicated nature of development and multiplication indicate that insect viruses are organisms with spherical

development stages and a rod shaped stage of relatively simple morphological structure.

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HEMAGGLUTINATION AS APPLIED TO THE STUDY OF VIRUS INFECTION

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There are 11 species of virus known to agglutinate red cells: *pneumotropic viruses*, influenza (human, swine), PVM (mouse pneumonia), and strain 1233 (human); *dermotropic viruses*, variola, vaccinia and ectromelia (pox virus of mice); *neurotropic viruses*, Theiler's virus (enteric and encephalitic disease of mice), encephalomyocarditis virus (endemic rat disease); *pantropic viruses*, fowl plague and Newcastle disease (generalized infections of fowl) and a virus with *glandular tropism*, mumps. It is obvious that this is a very heterogeneous group of agents in respect to the species of natural host, organ specificity, pathogenicity and virus particle size. The latter varies from very small to very large. Each virus has its own spectrum of red cell species with which it can combine. In a few cases the spectrum is narrow and largely confined to red cells from infectible species.

Among the viruses which cause hemagglutination there is a considerable disparity as to the type of reaction between virus and cell surface. In some cases the type of union is of general interest while in other cases the possibility exists that the union is of relatively little biological significance. In some cases the behavior of red cells and of the natural host cells toward the virus is remarkably similar, and in these instances it is felt that something may be learned from the red cell-virus interaction which may give clews as to the nature of events in the earliest stages of natural infection. One of the advantages of this type of study lies in the fact that *infection* of red cells does *not* occur and hence the interaction can be examined without interference from later stages of the growth cycle. The most serious disadvantage of such a model is that any information gained will probably be strictly limited to the earliest phases of the infectious process.

The foregoing viruses may be divided into two groups on the basis of the *type* of hemagglutinin. Variola, vaccinia and ectromelia comprise one group in which the hemagglutinin is separable from the active virus particle (Burnet and Boake, 1946). These viruses all form pox on their natural hosts, they are interrelated antigenically and the soluble hemagglutinin is thought to be a lipid, possibly cephalin (Stone, 1946). In the remainder of the viruses the hemagglutinin has not been dissociated from the virus particle. In the case of mumps, Newcastle disease and influenza viruses (MNI group) there is considerable evidence that the hemagglutinin is a part of the virus particle. Theiler's virus, encephalo-

myocarditis virus and fowl plague have been less thoroughly studied from this standpoint.

These viruses may also be divided into two groups in respect to their behavior toward receptors. In one group the virus-red cell combination may be dissociated only by changing the conditions under which adsorption took place (pH, salt concentration, etc.) and there is no evidence of any destruction of receptors by virus. All of the agents belong to this group except the MNI viruses, strain 1233 and fowl plague. In none of these cases has it been shown that the receptor site on the red cell is similar or related to the point of virus attachment of the natural host cell. Hence it is not clear whether red cell-virus reactions in any of these cases are specific in the sense of resembling part of a natural process.

The second group under this scheme (MNI viruses, 1233 and fowl plague) is specifically characterized by the ability of the adsorbed virus particles to *spontaneously* elute from cells and to exhaust the cell of virus receptors (Hirst, 1942, Burnet, 1945). Though the MNI viruses are heterogeneous in many respects their behavior toward receptors is such that they may be discussed as one group. Two main problems of biological interest are involved: (1) the importance of cell receptors in infection and (2) the role of receptor destruction in infection.

A wide variety of cells, both red cells of many species as well as the host cells in laboratory infections, readily adsorb viruses of the MNI group. Very likely the adsorbing group in all cases is of similar *general* character but not identical. Any of the viruses of this group can destroy the cell receptors for the entire group. In addition a number of bacteria manufacture an enzyme or enzyme complex which can destroy these same receptors, either on red cells or on living cells of other tissues (Burnet, McCrea, and Stone, 1946, Stone, 1947). Destroyed receptors are regenerated by living cells but not by red cells. Influenza viruses, among others, are adsorbed to and multiply in the cells of the chorioallantoic membrane of chick embryos. When the receptors of these cells are destroyed by a bacterial enzyme much larger amounts of virus are required to initiate an infection, so much more that it seems likely that influenza virus cannot infect a cell on which the receptors have been destroyed. This is the best evidence so far that receptors are necessary for infection.

The major effort in this field has been the study of the enzymatic or receptor-destroying role of these viruses. The idea that receptor destruction is an enzymatic process has gradually come to be accepted. The discovery of receptor-destroying activity in bacterial filtrates, the

destruction of inhibitors by viruses and the isolation of a receptor-like substance from red cells (also destroyed by viruses) have strengthened this assumption. The principal fact is that exposure of cells to virus leaves them devoid of receptors but the virus remains intact (Hirst, 1942).

For many years attempts were made to remove receptors from cells and then show that they reacted with viruses of this group. These results were unsuccessful because any attempt to combine receptors in solution with virus resulted in immediate destruction of the former. This difficulty was circumvented when it was discovered that the receptor-destroying capacity of a virus could be abolished without affecting the ability of the virus to *combine* with receptors (Anderson, 1948, Hirst 1948). This can now be readily done in a number of ways: treating the virus with heat, periodates, trypsin, etc. Such mildly injured virus combines in normal fashion with receptors, either on cells or in solution, but does not spontaneously elute. When such treated virus is exposed to an excess of receptor in solution, the virus becomes so saturated with receptors that it can no longer combine with or agglutinate red cells and hence soluble materials which have this effect are called inhibitors and virus which does not elute is called indicator virus.

One way to explain these facts is as follows: virus and red cells are bound to each other when agglutination occurs by mutually attractive forces of virus enzyme and receptor substrate. After contact has been made the cell substrate is destroyed, the bonding forces no longer exist and the virus diffuses away. When the virus is mildly injured by heat or other agents, the virus enzyme is sufficiently deformed so that it can no longer function enzymatically but enough configuration remains that "specific" adsorption between enzyme and substrate takes place. In the test for inhibitor, inhibitor competes with red cells receptors for the virus.

By the use of indicator viruses, inhibitors have been found in a wide variety of biological material: practically all mammalian organ emulsions, allantoic fluid, egg white, plasma, red cell extracts, ovarian cyst fluid, etc. There are obvious qualitative differences in the inhibitor from different sources and the chemistry of the active principle is in a primitive state. In one case, that of egg white, the inhibitor has been purified to a considerable extent and possesses very high biological activity (Gottschalk, and Lind, 1949). The active principle is at least in part polysaccharide and does not diffuse through cellophane membranes. In the presence of virus a substance in the fraction is broken down to smaller units which are dialyzable, and contain reducing substance but which have not been identified as yet. Satisfactory evidence

that this chemical breakdown is identical with inhibitor destruction has not yet been published.

On a biological level somewhat more is known about virus-receptor systems of which the receptor gradient sequence is a good example (Burnet, McCrea, and Stone, 1946). Red cells are exposed to a bacterial filtrate for different lengths of time so that one has a series of preparations with varying proportions of intact receptors. These cells are tested for their capacity to adsorb a series of virus strains of the MNI group. It is found that the capacity of cells to adsorb some strains is rapidly destroyed by the filtrate while other strains still adsorb well on cells that have had a prolonged exposure and have presumably lost most of their receptors. The arrangement of strains in the order in which their receptors disappear constitutes the gradient series and a very large number of strains can be so arranged, each a little different from the other. The same phenomenon has been demonstrated with *virus* destruction of receptors. From this and other experiments it appears probable that there are differences among cell receptors which are manifest in varied susceptibility to destruction and varied ability to combine with various strains.

Burnet and his collaborators in addition to finding differences in receptors have found differences in virus enzyme specificity which correlate perfectly with the virus gradient. If a number of strains are arranged in the order of their receptor gradient (1, 2, 3, 4, etc.), i.e. the order of increasing resistance of their cellular receptors to enzymatic destruction, then the following also occurs: when viruses of the gradient series are used for receptor destruction, strain 1 removes the receptors of itself alone, strain 2 removes receptors for 1 and 2, strain 3 for 1, 2 and 3, etc. This result implies a higher degree of specificity on the part of the virus enzyme. Such specificity was not present in other experiments (Hirst, 1950[a]), where it was found that each strain was capable of destroying the entire receptor complex provided the virus was present in sufficient concentration and allowed to act for a sufficient length of time. These two findings have not been reconciled. In the latter experiments the viruses which destroyed receptors with greatest speed also destroyed the receptor complex most completely, i.e. they were highest in the receptor gradient.

To explain the occurrence of a receptor gradient, Burnet postulated a single class of receptors buried at different depths in the cell surface and hence with varied ability to combine with virus and susceptibility to virus destruction. As will be seen below, this hypothesis will scarcely explain the behavior of inhibitors to virus action, and it seems at least equally valid to presume that the receptor site of the cell is a complex

of substances of different configurations, each part of the complex having different affinities for various strains and different susceptibilities to destruction. The pattern of such a complex may vary even among cells from different birds of the same species. The question of virus enzyme specificity is further amplified below.

Studies on the specificity of the virus enzymes and of substrate have been carried out with inhibitors from various sources. These have the advantage that rates of destruction can be more carefully measured than is possible with cell bound receptors (Hirst, 1950[b]). When material from a biological source (A) is tested for inhibitor content with a number of different strains (1, 2, 3, etc.) enormous variations (200 fold or more) in titer are found. Material from another source (B) also shows such variations and the amounts of different inhibitors found differ both relatively and absolutely from those of source A. (Inhibitor A may give titers of 1000 with strain 1 and 10 with strain 2, while B may have a titer of 50 with 1 and 500 with 2.) One way to interpret these findings is to assume that there are a number of active molecular species present in different proportions in the two samples. By measuring the rate of destruction of these inhibitors with different strains one obtains further information on this point:

(1) The inhibitors in preparation A are destroyed progressively by viruses and the inhibitor for strain 1 disappears at a faster rate than that for strain 2, etc., just as happened with red cell receptors.

(2) The order in which inhibitors disappear with virus action is characteristic for the source of inhibitor. However, this order may be completely inverted when material from another source is tested. (With preparation A the inhibitors for various strains may disappear in order 1, 2, 3, 4 and 5, while with B the order may be 5, 4, 1, 3, 2.)

(3) The order of inhibitor destruction is not, in general, affected by the strain used for destruction. If with preparation A, strain 5 inhibitor disappears more slowly than that of any other strain, the same holds even when strain 5 is used for destroying inhibitor activity. In this approach there is no strain specificity on the part of virus enzymes.

(4) There are marked differences in the speed with which different viruses destroy an inhibitor and in this there is some parallelism with the position of the strain in the receptor gradient.

(5) There are some exceptions to the statement about enzyme specificity in (3). Almost all strains, given sufficient time and when present in adequate concentration, can destroy the receptor complex completely (i.e. destroy the inhibitor active against any and all strains).

A few strains show a more limited ability. If strain 1 were one of these it might be found that it can destroy all strain 1 inhibitor in preparation A but cannot destroy completely the inhibitors for strains 2, 3, 4 etc. With preparation B however, strain 1 might be able to destroy the entire complex. This suggests that a restricted degree of enzyme specificity exists and also that there are qualitative differences in what is measured as strain 1 inhibitor between preparation of different sources.

In attempting to explain this evidence we may assume that there exists a spectrum of inhibitors or receptors, each with somewhat different affinities for various strains of this group and with different susceptibilities to destruction by virus enzymes. Virus enzymes attack this group of substrates in essentially similar fashion but with varying degrees of efficiency or speed. In a few cases an enzyme cannot attack parts of this substrate complex.

This picture of virus-receptor relationships would be more interesting if analogous systems could be found for other groups. There is an isolated but clear cut example of this in strain 1233 (Hirst, 1950b), an agent of respiratory infection in man, unrelated antigenically to the MNI group but which elutes spontaneously from red cells and has a receptor-inhibitor system quite unrelated to that which has been described for mumps, influenza and Newcastle disease. 1233 can destroy its own receptors in inhibitors but cannot attack those of the MNI group and vice-versa.

We must still face the somewhat speculative problem of what this system means in respect to the steps occurring in natural infection. The biological relationship of receptors and inhibitors is not clear though they are similar in a number of respects. Inhibitors play no obvious role in resistance to virus infection since they are readily liquidated by the virus and even when present in high concentration have only a weak effect on virus infectivity. Inhibitors may be chemically treated (Fazekas de St. Groth, 1949) so that virus is unable to split them, in which case the treated inhibitor becomes active in reducing the ability of virus to infect, apparently in a manner similar to that of virus antibody. There is no evidence that inhibitors behave this way in nature.

It has already been noted that virus receptors are produced by many living cells, including those susceptible to virus infection, and that intact receptors seem to be necessary for infection to take place. What role does receptor *destruction* play in infection? Since receptors occur on the surface of cells and since in red cell models the virus destroys this substance, it was suggested that the reaction provided the

necessary break in the cell membrane to permit virus entry. The evidence for this is very meager. It is known that the removal of receptors bares other, possibly deeper structures of the cell (Burnet and Anderson, 1947). It has been found that immediately after adsorption of virus to susceptible cells, the agent can be eluted by treating with bacterial enzyme preparations but that within a short time the virus disappears and is no longer elutable. The infectious process is accompanied by receptor (inhibitor) destruction (Schlesinger, 1950). All active strains of the MNI group possess the receptor destroying property. None of these facts eliminate the possibility that receptor destruction is an unnecessary side reaction of the infectious process. The strongest argument for the utility of this reaction for the virus economy is the unlikelihood of finding a vestigial process so prominent and uniformly present in an organism which is otherwise stripped below the essentials necessary for independent existence.

St. Groth has raised the only objections thus far to the foregoing hypothesis, but most of his conclusions seem too poorly documented to warrant a detailed discussion. His main argument is that periodate treated cells of the chorioallantoic membrane have receptors which cannot be destroyed by virus (Fazekas de St. Groth and Graham, 1949), yet the cells are readily infectible. This has not been substantiated by Schlesinger who found that under the prescribed conditions the receptors were, in fact, destroyed by virus (unpublished observations). The second approach (Fazekas de St. Groth, 1948) was more interesting than the first, though it would be helpful if the observations were confirmed and extended. He infected the cells of the chorioallantoic membrane and found that immediately after adsorption most of the adsorbed virus could be eluted through the use of bacterial filtrate. Within an hour or two the elutable virus had decreased to zero. Indicator virus, which cannot destroy receptors, "disappeared" (became non-elutable) at the same rate. This experiment, of course, does not deny the necessity of enzymatic action on receptors for the virus to enter the cell. The possibility should also be considered that the enzyme may function within the cell although it is not certain that inhibitor is found there.

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SOME SALIENT POINTS CONCERNING PLANT VIRUSES

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Chemical Nature of the Isolated Viruses.—(See Knight, *Ann. Rev. Microbiol,* 1949, 3,, 121.) The plant viruses isolated and highly purified thus far appear to consist solely of nucleic acid and protein combined to form specific nucleoproteins. Examples are tobacco mosaic virus, tobacco necrosis, tomato bushy stunt, Southern bean mosaic, turnip yellow mosaic, tobacco ringspot, and alfalfa mosaic.

The amounts of nucleic acid found in plant viruses range from 6% for the TMV group to about 40% for tobacco ringspot virus. All appear to be pentose nucleic acids. Most are bound rather firmly to their protein components.

The protein components of plant viruses appear to be acidic, rather than basic as in the case of classical sperm nucleoproteins. Analyses of 4 or 5 different viruses have revealed no unusual quantities or types of amino acids.

Crystallinity.—About a half-dozen of plant viruses (counting strains of TMV as only one instance) have been obtained in the crystalline state. Aside from the important implications concerning the fundamental nature of viruses associated with the first crystallization of a virus, no particular significance seems to attach to this property. At present, the so-called internal crystallinity of individual TMV rods, as revealed by X-ray analysis, appears to be of potentially greater significance than the orderly arrangement of numerous particles into paracrystalline aggregates. The X-ray studies indicate the presence of a repeat unit in the individual virus particles having dimensions of an approximately 11 A cube (Bernal and Fankuchen, *J. Gen. Physiol.*, 1941, *25*, 111). The demonstration of such units provides a definite basis for speculation concerning the possible mechanics of virus reproduction.

Size and Shape of Plant Viruses.—Each virus appears to possess a characteristic size and shape. The shapes revealed thus far by electron microscopy include globular, rod-like and filamentous forms. The sizes range from about 16 mμ for alfalfa mosaic virus to those of some other viruses whose one dimension, at least, is several hundred millimicrons. Some speculation is occasioned in the case of TMV by the occurrence of rods of different lengths in preparations of this virus. However, the

available evidence favors the association of infectious quality with the approximately 15 x 300 mμ rod. The longer rods appear to arise by aggregation and the shorter ones by various forms of disruption of the 300 mμ rods.

Infectious Unit of TMV.—Several million particles of TMV, or of other plant viruses, are usually required to cause an infection, but this is largely attributed to the inefficiency of existing techniques in introducing the viral particles into the susceptible cells. A mathematical analysis by Lauffer and Price indicates that one viral particle is probably sufficient to initiate infection *(Arch. Biochem.*, 1945, *8*, 449).

Relationship of TMV to Host.—Apparently identical TMV is obtained from serologically totally unrelated plants such as Turkish tobacco and phlox. (Gaw and Stanley, *J. Biol. Chem.*, 1947, *157*, 765.) The virus in each instance has, until the recent work of Wildman, appeared to be a foreign entity quite distinct serologically and otherwise from the normal constituents of the host plants. Wildman's investigations, if substantiated, would indicate some relationship between macromolecular cytoplasmic constituents and TMV.

Relationship of Plant Viruses to Animal Viruses.—The viruses of all types investigated thus far possess the chemical common denominator of nucleic acid and protein. The most consistent difference between animal and plant viruses seems to be the invariable association of lipid with animal viruses. Of the animal viruses investigated, the Shope papilloma virus most closely resembles the plant viruses in composition since it is almost solely nucleoprotein. No obviously successful cultivation of a plant virus in higher animals or the reverse has been reported. However, the results of Black *(Phytopath*, 1949, *39*, 2,) strongly support the multiplication of plant viruses in insects, and suggest the possibility that some plant virus may yet be demonstrated to multiply in some vertebrate host.

Mutation of Plant Viruses.—Mutation is common among plant viruses although the frequency of mutation seems to vary considerably with different viruses. No exhaustive studies have been made on the frequency of mutation of TMV, but Kunkel found that under some circumstances the figure may be of the order of 0.5 per cent. (Publication No. 12, *A.A.A.S.*, 22-27, 1940). The mutation of plant viruses results in new strains. Mutation is sometimes followed directly. In other instances, strain relationship between 2 viruses is established logically although somewhat arbitrarily by demonstration of (see, for instance, Holmes, *Phytopathology*, 1941, *31*, 1089):

1. A similarity in host range
2. Coincidence of general chemical and physical properties
3. Possession of common size and shape
4. Positive cross-interference tests
5. Positive serological cross reactions
6. Similar response to genetic change in host
7. Similar method of transmission.

The more of the above criteria satisfied, the stronger is the argument for strain relationship. On the other hand, failure to satisfy one of the criteria need not preclude strain relationship.

Some strains of TMV have been shown to differ in protein composition.

Relation of Chemical Structure to Biological Activity.—A number of derivatives of TMV have been synthesized in which known chemical reagents have been coupled to active groups of the virus. In some cases it was found that 70% of the amino groups and 20% of the phenol plus indole groups could be substituted before measurable loss of biological activity was observed. Further substitution caused loss of activity. Subsequent studies on strains of TMV have suggested that the biological activity probably does not reside in the surface active groups *per se* but rather involves some more subtle and at present obscure structural characteristic of the virus.

The importance of nucleic acid in plant virus reproduction has been emphasized by the findings of Markham and associates with turnip yellow mosaic virus. Preparations of this virus were obtained containing essentially 2 components, one a macromolecular protein and the second, a macromolecular nucleoprotein. The protein constituent of the nucleoprotein appeared to be identical with the free macromolecular protein, but only the nucleoprotein was infective (*Parasitology*, 1949, *39*, 330).

PRECURSORS OF BACTERIOPHAGE NITROGEN AND CARBON*

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It has been known for some time that bacteriophage-infected cells require an exogenous source of nitrogen and oxidizable carbon for growth of virus (Spizizen, 1943). These early results imply that nitrogen and carbon of the medium are assimilated for synthesis of bacteriophage but do not demonstrate the incorporation of elements of the medium into the phage nor reveal the intermediate pathways or precursors. Isotopic investigations of workers in this laboratory (Kozloff, Knowlton, Putnam and Evans, 1950; Barry, Gollub-Banks and Koch, 1950), which are reported herein, disclose the sources of bacteriophage nitrogen and demonstrate a substantial transfer of purines from the host to the virus. While the medium is the ultimate source of most of the virus N, P, and C, the investigation has been chiefly concerned with the nature of the bacterial contribution. The results indicate that the protein and nucleic acid of the virus are in part derived from bacterial precursors and that a substantial portion of the bacteriophage DNA arises from bacterial DNA.

The principles and conduct of the experiments have already been described (Kozloff and Putnam, 1950). Bacteriophage was grown by multiple infection on labeled host cells or labeled medium (synthetic lactate), purified by differential centrifugation, and characterized in the analytical ultracentrifuge and by infectivity measurements. In most instances the purified phage was partitioned into nucleic acid and protein and the nucleic acid was hydrolyzed to give purines and pyrimidines. The purines were separated chemically, and the adenine or guanine crystallized to constant radioactivity with added carrier or were isolated by column chromatography without added carrier. Thymine was purified by sublimation. In several instances the protein was hydrolyzed and the acidic, basic, and neutral amino acid fractions were separated by ion exchange columns. In a few experiments the medium was labeled with $P^{32}O_4^{\equiv}$, or with $N^{15}H_4Cl$, but in most cases the host was labeled. The bacteria were labeled as follows: a) fully labeled with P^{32} or N^{15} (by growth on isotopic media so that all P or N fractions were equally labeled), b) differentially labeled with P^{32} or N^{15} (grown in isotopic medium as in (a) but the washed bacteria then allowed to metabolize in growth limiting medium (for P^{32}) or to

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undergo one or two divisions (for N^{15}) so that the P or N fractions were now equally labeled), c) specifically labeled with C^{14} in the purine carbon (by growth in medium containing isotopic adenine), or d) doubly labeled with P^{32} and N^{15} (by growth in medium containing both isotopes).

Origin of Virus Phosphorus.—In agreement with Cohen (1948), already published work (Kozloff and Putnam, 1950) on the origin of virus P has shown: 1) There is after infection a net synthesis of desoxyribonucleic acid (DNA); and 2) The inorganic phosphate of the medium is the ultimate source of 70-80% of the P of T2, T4, and T6 bacteriophages. Further, our work has shown 3) Quantitatively, only a small fraction of the bacterial P is utilized for virus synthesis, and 4) The latter fraction is apparently not acid soluble P but appears to be a stable and possibly specific precursor of phage DNA. From this work the hypothesis was advanced (Kozloff and Putnam, 1950) that much of the bacterial DNA is either transferred intact or in degraded form for synthesis of some of the phage DNA. The experiments with N^{15} and C^{14} are compatible with this hypothesis but do not yet rigorously exclude bacterial RNA N, P or purines as a source of *some* of the phage DNA.

Origin of Virus Nitrogen.—In 14 experiments, summarized in Table I, it was found that the medium is the source of the major part of virus N, but that also bacterial N appears in both phage nucleic acid and protein. Quantitatively, only a small fraction of the bacterial N ends up in the phage. More bacterial N was found in the virus nucleic acid than in the virus protein. Thus, for whole phage, from 11.6% to 38.8% of the N was derived from the host with a mean value of about 20%; for the virus nucleic acid, from 16-43% of the N came from the host, and for phage protein, from 6 to 27% of the N was bacterial N. In all cases but one (Expt. 14) (performed in broth) more virus nucleic acid N came from the host than did virus protein N.

The above summary emphasizes the variability of the proportion of host N in the virus but does not show the effects of yield of virus, composition of the medium, or time of liberation on the isotope distribution in the virus. All these effects were investigated, and in Table I the experiments are listed in order of increasing average yield of virus per infected bacterial cell. The data show an inverse relationship of the per cent of phage protein N derived from the host with yield of virus but no correlation of the latter factor with the per cent of phage nucleic acid N derived from the host. These results were first estab-

TABLE I

RELATION OF THE YIELD OF PHAGE PER BACTERIAL CELL TO THE AMOUNT OF BACTERIAL N CONTRIBUTED TO PHAGE PROTEIN AND NUCLEIC ACID

Experiment*	Yield of phage per bacterium	% of Total phage N derived from host	% of Phage protein N derived from host	% of Phage nucleic acid N derived from host
9	17	38.8	26.6	42.7
14	33	22.1	21.3	16.9
4	50	28.0	21.5	37.2
6	50	28.9	17.6	37.1
10	60	31.1	12.7	43.2
7	80	26.9	17.4	38.1
5	117	20.9	13.2	24.9
13	117	13.1	8.2	16.9
11	117	26.3	10.5	38.2
3	160	20.6	15.1	26.1
8	163	25.9	11.0	37.9
1	173	18.9	8.8	33.3
2	215	21.4	7.8	27.7
12	287	11.6	5.7	16.6

*Arranged in order of increasing yield.

lished by experiments in which phage samples were taken and analyzed at different times after infection (Table II).

In any given experiment the N^{15} content of the phage nucleic acid is nearly independent of the time of sampling, but the N^{15} content of the phage protein falls with time. The differential utilization of host N is most marked in the later time intervals. The data are in accord with the hypothesis that the phage nucleic acid N is partially derived from a stable precursor (possibly DNA) and the phage protein N is

TABLE II

KINETIC STUDY OF THE SOURCES OF VIRUS NITROGEN

Experiment	Per Cent of N Derived from Host*					
	I			II		
Incubation Time (hours)	5.5	7	24	3	5	24
Total Phage N	28.9	26.9	25.9	38.3	31.1	26.3
Phage Nucleic Acid N	37.1	38.1	37.9	42.7	43.2	38.2
Phage Protein N	17.6	17.4	11.0	26.6	12.7	10.5

* $\frac{\text{Atom \% excess } N^{15} \text{ in virus N}}{\text{Atom \% excess } N^{15} \text{ in bacteria prior to infection}} \times 100$

derived from an exhaustible substrate. On the other hand, although the standard medium contains only 0.01% NH_4Cl (the minimal amount for good phage growth in resuspended bacteria), raising the N content of the medium over a 10-fold range or supplementation of the medium gave no clear effect on either the isotope degree of incorporation or the distribution of the isotope in the phage.

Host N^{15} was found in all the substances isolated from the phage: protein, nucleic acid, the adenine, guanine, thymine, and cytosine of the nucleic acid, and the acidic, basic and neutral amino acid fractions of the hydrolyzed protein. No significant difference appeared in the isotope distribution in the three amino acid fractions, but significant differences were noted in the distribution of N^{15} among the purines and pyrimidines. As shown in Table III guanine was labeled to a greater extent than adenine. The high incorporation in the thymine is noteworthy since bacterial DNA is the only appreciable host source of this base.

The simultaneous transfer of bacterial N and P to phage nucleic

TABLE III

THE CONTRIBUTION OF BACTERIAL N TO PHAGE NUCLEIC ACID BASES

Phage Fractions	Experiment 8 N^{15} Labeled Bacteria	Experiment 13 N^{15} Labeled Bacteria
	% of N derived from host bacterium	% of N derived from host bacterium
Nucleic acid N	16.9	16.0
Purine N	16.9	17.3
Adenine N	14.8	14.0
Guanine N	18.8	21.8
Thymine N	15.9	14.5
"Cytosine N"	17.8	14.7

TABLE IV

ISOTOPE CONTENT OF NUCLEIC ACID FROM T6 BACTERIOPHAGE GROWN ON *E. COLI* CONTAINING N^{15} AND P^{32}

Expt. No.	Bacteria		Virus NA		Relative Isotope Content		Ratio of Bacterial Contribution N/P
	N^{15} atom % excess	Specific radioactivity c.p.m. per γ P	N^{15} atom % excess	Specific radioactivity c.p.m. per γ P	$\frac{\text{Virus NA N}}{\text{Bacterial N}}$	$\frac{\text{Virus NA P}}{\text{Bacterial P}}$	
					per cent	per cent	
3	9.64	201	3.59	57.4	37.2	28.5	1.31
10	10.5	252	2.74	45.6	26.1	18.1	1.44

acid was investigated using doubly labeled host cells. When the bacteria were labeled with N^{15} and P^{32},

$$\frac{\%\text{ of virus nucleic acid N from host}}{\%\text{ of virus nucleic acid P from host}} = 1.3 \text{ to } 1.4 \text{ (Table IV).}$$

While the results summarized in the discussions and conclusions indicate that bacterial nucleic acids are the precursors of some of the phage DNA, the ratio in the double labeling experiments is significantly different from unity—the value expected for transfer of intact host DNA to virus DNA (provided host and virus each has only one and the same kind of DNA). This suggests utilization of nucleotides or possibly of larger fragments of host nucleic acid for virus synthesis.

Transfer of Purines and Pyrimidines from Host to Virus.—Radioactive adenine has been isolated from *E. coli* grown on ammonium lactate medium supplemented with $NaHC^{14}O_3$ and has been used to label the host. Bacteria were specifically labeled in the purine fraction by culturing them on lactate medium supplemented with C^{14}-labeled adenine. In this case both the adenine and the guanine of the cells are equally labeled and account for all the radioactivity of the bacteria. Phage was grown on the washed labeled cells, and the purines of the phage were isolated.

TABLE V

TRANSFER OF LABELED PURINES FROM BACTERIAL HOST TO BACTERIOPHAGE PROGENY

Expt.	Material	Thick Sample Count		Per Cent of Phage Purine from Host
		Bacteria	Phage	
		counts	per minute	
I	Purine carbon	4,250	2,230	52%
II	DNA adenine	56,500*	7,920*	14%
	DNA guanine	59,000*	11,900*	20%

*Quantities contained in 10^{12} *E. coli* cells and resulting phage:
Bacterial DNA—adenine 1.08 mg., guanine 1.78 mg.
Phage DNA—adenine 3.68 mg., guanine 2.28 mg.

In the first experiment (Table V) a large weight of carrier adenine

or guanine had to be added to a minute amount of the phage purine with the result that the specific activity of each of the purines could not be determined. In this experiment, with low phage yield in minimal medium, about 50% of the phage purines were derived from host material assimilated prior to infection. In the second experiment a minute amount of chromatographically pure labeled adenine was incorporated in the host cells. The washed cells were infected in N-rich medium (0.1% NH_4Cl). The adenine and guanine of the resulting virus were isolated chromatographically (presumably recovered quantitatively), the amounts determined spectrophotometrically, and the activity measured after the addition of carrier. The data of Table V indicate that 14% of the virus adenine and 20% of the virus guanine arose from material in the host prior to infection. It is noteworthy that the *total radioactivity* of the phage adenine (wt. in mg. x thick sample count) is almost equivalent to the total radioactivity of the phage guanine. The preliminary data on recoveries indicate that there is a net synthesis of DNA purines (total purine of liberated phage equals twice the DNA purines of the host cells) and also suggest a remarkable difference in the chemical composition of phage DNA and bacterial DNA.

DISCUSSION AND CONCLUSIONS

1. Direct isotopic investigation confirms that constituents still in the medium at the time of infection are the ultimate source of most of the N, P and C of bacteriophage. This is incompatible with the precursor theory of virus reproduction in its crudest form.

2. Quantitatively, only a small fraction of the bacterial P (about one-twelfth) and of the bacterial N (about one-twentieth) is used up for phage synthesis.

3. Considering now the precursors of protein N, a) Phage protein synthesis is reported to precede phage DNA synthesis (Cohen, 1948), but phage DNA from labeled cells contains more isotope than does phage protein, b) Bacterial N incorporation into phage diminishes with time of sampling following an exhaustible substrate type of curve despite the ample supply of isotope in the host protein, c) Bacterial N appears about equally in all amino acid fractions, and d) Enzyme adaptation in *E. coli* ceases on phage infection (Monod and Wollman, 1947); infected bacteria no longer divide or grow, they produce only nucleoprotein equivalent to phage (Cohen, 1948), and e) The isotope content of the debris is 70% that of the infected bacterium. The facts c, d, and e suggest that protein turnover ceases in infected bacteria and proteolysis follows rather than precedes elaboration of the phage. Hence,

phage protein is built up from simple substrates in rapid equilibrium with the ammonia of the medium. Presumably, these are found in the acid soluble N fraction which is adequate to account for the observed incorporation of bacterial N into phage protein N.

4. Considering now the precursors of phage DNA: a) P, N, and C of the host all appear in substantial amounts in the virus DNA, and in synthetic medium host N always appears both in greater proportions and greater amounts in phage DNA than in phage protein, b) Bacterial RNA does not turn over after virus infection (Cohen, 1948), c) Host purines are transferred intact to phage DNA (intact since the C^{14} is found only in the purine carbon of the host and the virus), d) Phage thymine N originates in the host to an equal extent with phage purine N though there is a much larger reservoir of purines (RNA+DNA) in the bacteria than of thymine (DNA only), and e) There is sufficient N, P, purines, and thymine in the bacterial DNA of the average cell to account for all of the bacterial contribution of these substances to virus DNA, although there is insufficient purine in the initially acid soluble fraction to account for the total of this transfer. Hence, complex substrates are utilized for phage DNA synthesis. These may be simple purines but more probably are nucleosides, nucleotides, or polynucleotide fragments. However, intact bacterial DNA is probably not transferred to phage DNA (excluded by the double labeling experiment, the different ratios of base transfer, and the tentative conclusion that the two DNA's have different composition). Sources of these substrates may be the initial acid soluble pool of nucleosides, etc., RNA, and DNA. At present, the insufficiency of the acid soluble pool and the inertness of the RNA seem to eliminate these fractions as major precursors, but the sufficiency of the DNA, the utilization of thymine in equal amounts with purines, and the lack of effect of acid soluble bacterial P turnover on P utilization for virus synthesis all appear to implicate bacterial DNA as the precursor of a substantial fraction or fragment of virus DNA. Presumably, this bacterial DNA is first degraded and then rebuilt into phage DNA.

The finding that the host is the source of some of the N, P, and C of the phage does not minimize the significance of the net synthesis of desoxyribonucleoprotein in infected bacteria but rather emphasizes the active participation of the host. It is clear that the simple substrates of the medium must be absorbed by the infected bacterium, elaborated into more complex units (amino acids, purines, etc.) presumably by the synthetic mechanisms of the host, the latter are then combined into nucleoprotein molded into specific configurations possibly by intervention of genetic governors of the phage. Since previous genetic

and biochemical studies of the fate of the infecting virus particle have shown that it loses its identity and merges with the host, protein and nucleic acid synthesis probably occurs around segregated subunits acting as templates and the products of these are then assembled into complete virus particles prior to lysis of the host.

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RECENT STUDIES ON THE HOMOGENEITY OF TOBACCO MOSAIC VIRUS

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The homogeneity of tobacco mosaic virus particles has been the subject of much study and controversy. Considerable attention has been directed toward the establishment of the identity of infectivity with the large rod-like particles isolated from virus diseased tobacco plants. One point of view has held that the virus particles have different lengths, varying from about 35 mμ to 1000 mμ, any one of which or most of which possess infectivity. On the other hand, it has been contended that there is a minimum length particle of about 300 mμ which possesses this biological activity. These points of view have been adequately reviewed in the past (Pirie, 1945; Lauffer, Price, and Petre, 1949); therefore, this discussion will concern itself with recent unpublished experiments designed to provide useful information relative to this problem.

Since it is now common practice to isolate tobacco mosaic virus and other viruses by a series of cycles of alternate high- and low-speed centrifugation, we might profitably consider the method briefly. It is well known that the so-called differential centrifugation process is inefficient as a method of fractionating particles of differing sedimentation rates. It is to be expected, therefore, that the final solution of "purified" virus obtained with the aid of conventional preparative centrifuges would contain a distribution of particles which is related to the distribution in the original juice expressed from the plants. Differences would arise due to alterations of the particles caused by aggregation and rupture, or denaturation and insolubilization of the particles during the isolation procedure. Particles having sedimentation rates much larger and much smaller than those of the principal components would be eliminated in the course of the cycles of alternate high- and low-speed centrifugation.

If, now, the final preparation contained particles of different sizes it would be important to determine with a convection-free analytical ultracentrifuge the sedimentation rate of the infectious principle in an effort to correlate biological activity with a specific particle. Lauffer (1943), using the separation cell in the ultracentrifuge, demonstrated that the infectious agent had a sedimentation constant which was

almost equal to that observed for the principal component composed of particles with lengths calculated to be about 270 mμ.

On the other hand, we might test the assumption that the particles have different lengths by attempting to attach limits to the variation in particle lengths in a given solution. This approach has been followed by Williams and co-workers* (in press) using new electron microscope techniques and by Schachman (in press) using the ultracentrifuge. The electron micrographs were made on aliquots of a solution obtained by heating the expressed juice to 56°C. The ultracentrifuge studies were made on solutions prepared in the conventional manner of differential centrifugation. Since one method is used to study particles in the dry state and the other is used to examine collections of particles in solution, it is interesting to compare the results.

By means of special techniques Williams and co-workers were able to examine micro-drops of dilute solutions in a manner which permitted them to measure every particle present in a given drop. Most electron micrographs of tobacco mosaic virus show a large percentage of particles with lengths about 300 mμ and a small percentage of shorter particles of varying lengths as well as a small number of longer particles. In their studies, Williams and co-workers found that 70% of the particles seen in the micrographs had a length close to 300 mμ. Since each drop pattern contained only about 15 particles they were able to measure all of the particles, and they found that the particles of abnormal length can be summed to equal precisely a small whole-number multiple of the value, 300 mμ. This work required the use of very dilute solutions and the study of many individual patterns, so that the summing of particles would be confined to only a few particles in a given field. Williams and co-workers concluded that over 96% of the virus particles exist either as monomers of length about 300 mμ or as multiples of two or three times this length. Presumably then, the short particles seen in most electron micrographs arise from rupture of 300 mμ length particles when a drop containing these particles is dried preparatory to examination in the electron microscope.

Despite the limitations of the ultracentrifuge in the study of rod-like particles, it seemed of interest to re-examine the problem of the homogeneity of the virus particles with respect to length in the light of the progress in the experimental techniques for the purification and

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* The author is indebted to Dr. Robley C. Williams for his permission to use these data before their publication.

study of the virus. Furthermore, it was important to study solutions of virus particles as a common study to that performed by Williams and co-workers on dried specimens. Boundary spreading experiments show that our recent preparations are much more homogeneous than those studied earlier by others and by ourselves. The enhanced homogeneity of these preparations is due presumably to the salt concentration used during the isolation and study of the virus. Since the method of purification involved the same type of differential centrifugation as that used previously, particles of ¾ the length of the normal particles would not have been eliminated. Because of the inadequacy of accurate diffusion data it cannot be stated categorically from the spreading experiments that the virus is or is not homogeneous. Therefore, another approach was used. It happens that a physical anomaly in ultracentrifugation magnifies the effect of trailing components in ultracentrifuge patterns (Johnston and Ogston, 1946). Thus a 50-50 mixture of tobacco mosaic virus and a smaller particle might appear to be a 10-90 mixture. This effect seemed to provide a means of detecting small amounts of shorter particles in the presence of large amounts of virus particles. A model substance of particles with lengths about ¾ that of the virus was obtained by chemical degradation. Mixtures of it and virus were then studied in the ultracentrifuge. The results showed clearly that two parts of the ¾ length particle could be detected in the presence of 98 parts of virus. It can, therefore, be stated that our best preparations of tobacco mosaic virus can not contain more than about 1% of ¾ length particles. This test implies that our mixed "impurity" must itself be relatively homogeneous, otherwise we would not detect it. Since most electron micrographs do show ¾ length particles in quantities greater than 1%, it would appear that they arise from breakage of the uniform virus particles.

In closing, it is important to note that both of these studies were made on preparations obtained from the expressed juice of diseased tobacco plants. Other workers obtain greater yields of virus due to additional treatment of the pulp remaining after expressing the plant juice. Studies of the type described here must be performed on the virus solutions prepared by these other methods before a detailed statement of comparison can be made.

In summary then, the recent work using new electron microscope techniques and ultracentrifugal analyses indicates that the virus particles obtained from the expressed juice of diseased tobacco plants possess a remarkable degree of uniformity with respect to length.

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INTERFERENCE BETWEEN ANIMAL-PATHOGENIC VIRUSES

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It has become general practice to classify as interference phenomena all instances in which exposure to one of a pair of viruses renders a host refractory to the disease-producing effects of the other or unable to support its multiplication, and in which this acquired refractoriness cannot be readily explained as due to conventional specific immune reactions. According to this broad criterion, i.e. without regard for possible diversities in the underlying mechanisms, one can say that mutual interference between animal-pathogenic viruses is (a) not dependent upon immunological or even obvious biological relationship between the two viruses involved, and (b) not restricted, as is "mutual exclusion" between phage strains (Delbrück and Luria, 1942), to unrelated combinations. Henle has just written an exhaustive review of the entire subject (Henle, W. 1950), in which he emphasizes the varying degrees of relationship between viruses which do or do not interfere with each other. In this paper, an attempt will be made to reduce the problem to the level of the single infected cell and to form the basis for further discussion in which some analogies and some contrasts to the phage-bacterium system might be brought out. Such a limitation in scope calls for the following reservations:

(a) "Interference" between immunologically related viruses may be due to specific immune mechanisms, provided, of course, the animal host is capable of antibody production. Recent findings on the rapidity and efficacy of local antibody response, stimulated in immunized animals by the antigenic "booster" of the challenge dose (Schlesinger, 1949a), suggest the possibility that a specific immune response might play a role even in those cases in which the "interfering" dose had not led to the production of measurable antibody by the time the related challenge dose was given. The interpretation of such observations, i.e. the choice between interference and specific immunity, should be based on determination of the *additive* antigenic effect of the two doses. Obviously this reservation does not apply in instances of interference between immunologically related viruses which have been studied in hosts incapable of antibody production, i.e. the embryonated egg or embryonic tissue culture media.

(b) Interference has been observed in a variety of extremely dissimilar host-virus systems (see Henle, W. 1950), and it is not always known what cells are infected by the viruses. If the cells are identified,

they are not available in any state resembling pure culture. Even a fairly homogeneous, single-type cell surface structure like the endothelium of the chorioallantoic membrane cannot readily be separated from other structures making up the various layers of the membrane. Each cell lives in anatomical and physiological dependence on others and not as an individual entity. Hence, it is impossible, with any degree of assurance, to separate extracellular from intracellular phases of infection.

(c) The conjecture that interfering viruses have in common an affinity for the same type of cell has in many cases little experimental basis. Histopathological lesions found in infected organs and resulting functional impairments may well be end- or by-products of the infectious process, and it is conceivable that the cells involved in the mechanism of interference may show no abnormalities at all.

Bearing these reservations in mind, we have no choice, in view of the intimate association between virus and host cells, but to assume that it is the known or unknown susceptible cell which is the common denominator between the interfering and the interfered-with virus. We have to make this assumption for a relatively simple host organ, such as the entodermal epithelium of the allantoic membrane, as well as for a complex mammalian organ like the mouse brain which contains such a variety of cellular elements other than neurons. The quantitative aspects of interference are in keeping with the assumption that it is effected in conjunction with the *individual* host cell. Thus, while active influenza viruses of types A and B are capable of mutual interference in the allantoic sac of chick embryos under certain conditions of dosage and timing (Ziegler and Horsfall, 1944), it is also possible to propagate a mixture of both serially from egg to egg if the inoculum is properly diluted and adjusted so as to contain comparable amounts of both viruses (Sugg and Magill, 1948). The significance of this latter observation, insofar as it might seem to conflict with the observed interference between the two viruses, would depend on the unequivocal demonstration that both multiply in the same individual cell. That has not been shown either for this combination or for the mumps- or PVM-influenza combinations for which dual infections in eggs or mice have been demonstrated under some conditions (Gingsberg and Horsfall, 1949), while under others, mumps and influenza viruses have given reciprocal interference (mentioned by Henle, W., 1950). The only convincing instances of simultaneous infection of *single cells* by two unrelated animal viruses so far recorded appear to be those in which such cells showed both intranuclear and intracytoplasmic inclusion

bodies characteristic of the two infecting viruses (Anderson, K. 1942, Syverton and Berry, 1947).

In most examples of interference between animal-pathogenic viruses which have been described in the literature, the interfering agent has the actual or potential ability to impair the integrity of certain cells in a characteristic manner*. This generalization applies regardless of whether the interfering virus is active or "inactivated." In either case, the intensity of the inhibitory effect is proportional to the quantity of interfering virus to which the susceptible tissue is exposed, and may be related to its ability to interact in some specific way with the cells of that tissue. We can test the validity of this broad statement by examining specific instances of interference.

Example 1: With actively multiplying virus, the interfering capacity increases with progression of the infectious process.—This is exemplified by the interference, in the mouse brain, between Theiler virus (T.O.) and Western equine encephalitis (W.E.E.) virus, two totally unrelated agents (Schlesinger, Olitsky and Morgan, 1944a). Mice inoculated intracerebrally with 100 ID_{50} of T.O. develop paralysis of the extremities after 9 to 14 days. If they are reinoculated during the incubation period with graded amounts of W.E.E.—which produces fulminating encephalitis with death after 2 to 3 days in control mice—they show a gradually increasing degree of refractoriness to the latter. By the 8th day, they resist as much as 10^7 LD_{50} of W.E.E. The course of the infection with T.O. virus is, however, in no way affected by the intervening inoculation of the more virulent of the two.

Example 2: With actively multiplying virus, the degree of interference wears off after arrest of the interfering infection.—Vaccination of laboratory animals with formalin-inactivated *Western* E.E. virus leads to immunity to W.E.E., but not to the immunologically distinct *Eastern* E.E. virus. However, the specific immunity is not absolute, and intracerebral challenge with W.E.E. may result in an abortive encephalitis during which some virus multiplication may take place. This abortive infection renders the animals temporarily refractory to subsequent infection with Eastern E.E. or vesicular stomatitis virus. After 2 to 3 weeks, full susceptibility to the heterologous agents is restored, while specific immunity persists (Schlesinger, Olitsky and Morgan, 1944a,b).

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* In speaking of this ability as "potential," I mean that most "non-pathogenic" interfering viruses have either the inherent capacity of being "adapted" to the host tissue in which they interfere, or are derivatives of parent strains which *are* pathogenic for that tissue.

Example 3: If active virus is incapable of multiplication, but can interfere, large quantities are required which may impair the integrity of cells.—The most striking example in this category is the interference, again in the mouse brain, between non-neurotropic strains of influenza virus and certain neurotropic viruses, e.g. W.E.E. Vilches and Hirst (1947) have shown that relatively large amounts of influenza virus have to be given for significant interference. That influenza virus is not entirely innocuous for the mouse brain is clear from Henle's studies on its "toxicity" after intracerebral inoculation of high doses (Henle and Henle, 1946). At least one strain, the W.S. of type A influenza virus is "potentially pathogenic" in that it can be adapted to the mouse brain so that it multiplies there and produces typical encephalitis (Stuart-Harris 1939, Francis 1940). Further pertinent effects of intracerebral inoculation of non-neurotropic influenza viruses have recently been observed in our laboratory, and will be described below in a discussion of the mechanism of interference on the basis of the most amenable study object, i.e., influenza virus.

Example 4: Inactivated interfering virus retains the ability to impair the integrity of the cell, and large quantities are required.—Ultraviolet-irradiated influenza virus of one type, if given in large amounts, can interfere in the egg with the propagation of active virus of another type (Henle and Henle 1943, 1944a,b, 1945, Ziegler, Lavin and Horsfall, 1944). If ultraviolet-irradiated influenza virus is injected in high concentration, it significantly inhibits the growth of the allantoic membrane and also of the embryo itself (Henle, Henle and Kirber, 1947). This growth-inhibiting action is ultraviolet-resistant to about the same extent as the ability of the virus to interfere in the egg with active homologous or heterologous influenza virus. The fact that interference by irradiated influenza virus also requires the administration of large amounts again suggests that it is related to its residual ability to affect the normal function of the host cell. Similarly, bacteriophage which after irradiation retains its interfering capacity inhibits bacterial multiplication (Luria and Delbrück, 1942).

While these examples are chosen arbitrarily from a large number of observations (see Henle 1950), it seems that no instance of interference has been described in the literature which would contradict in essential points the general suggestions which they serve to illustrate.

These statements do not say anything specific concerning the mechanism underlying interference. It may be said without much argument that all evidence points to the virus particle itself, whether active or inactivated, as the essential factor.

Thus, even after inactivation with ultraviolet light, influenza virus retains its basic identity (Henle and Henle, 1947). While it has lost its self-reproducing capacity, it retains its ability to be adsorbed onto and eluted from erythrocytes, to be adsorbed on the allantoic epithelium, to inhibit the growth of the allantoic membrane, and to act as specific antigen in vivo or in vitro. The fact that irradiated virus remains elutable from red cells suggests that it is capable of destroying the red cell receptors. Since many similarities exist between these receptors on red cells and hemagglutinin-inhibitory (VHI) substances extractable from erythrocytes (Friedewald et al., 1947, deBurgh et al., 1948), normal serum (Francis 1947, Hirst 1948) and various fluids and tissues of susceptible species (Friedewald et al., 1947, Svedmyr 1948a,b, Hardy and Horsfall 1948, Schlesinger 1949b, unpublished findings), it may be assumed that these inhibitors are cellular receptors in solution. Active influenza virus is capable of destroying these substances in vitro and also in the infected host, and it is likely, in view of its elutability, that irradiated virus can do the same. Henle's data on the reduction in virus-adsorbing capacity of the allantoic sac after inoculation of irradiated virus (Henle, Henle and Kirber, 1947) are suggestive in that direction, but could as well be due to persistent attachment of the irradiated virus to cell receptors. No attempt has as yet been made to do direct receptor (VHI) titrations either on normal serum or on extracts of membranes after exposure to irradiated virus. It would be of interest to clear up this point, not so much perhaps because of any suspicion that receptor destruction or blockade *per se* might be responsible for interference, but because of the still valid assumption that receptor destruction is an essential prerequisite for virus entry into the cell (Hirst 1943).

The hypothesis of receptor destruction or blockade as the mechanism underlying interference was formulated when the phenomenon of interference between inactive influenza virus and active homologous or heterologous viruses was first observed by Henle et al. (1943), and by Ziegler et al. (1944). It lost much of its validity—at least as the *only* factor—when it was shown that (a) the adsorptive capacity of the allantoic sac was not completely blocked by interfering quantities of irradiated virus (Henle, Henle and Kirber 1947), and (b) the interfering capacity of the virus was more UV-sensitive than the capacity of being adsorbed by the allantoic epithelium (Henle and Henle 1947). Other evidence in the same direction was obtained in Burnet's and in our laboratory when the "interfering" effect of cholera vibrio filtrates (receptor-destroying enzyme or RDE) was compared with that of virus. The enzyme, while much more effective than influenza virus in destroying receptors of the respiratory epithelium (Fazekas de St. Groth

1948), of the allantoic sac (Stone 1948, Schlesinger, to be published), or of tissue culture media (to be published), is proportionately far less effective in blocking the susceptibility of these tissues to infection with active virus. We have also tried to determine whether the interference between influenza and encephalitic viruses (Vilches and Hirst 1947) might possibly be due to the destruction of a common receptor. It was found that (a) W.E.E. virus has no effect at all on the VHI titer of mouse brain extract even on prolonged incubation at 37°C; (b) the VHI titer of brains from mice dead of W.E.E. infection is identical with that of normal brains; (c) W.E.E. virus grows out to full titer in minced embryonic tissue even after all demonstrable influenza receptors (VHI) have been completely destroyed by receptor destroying enzyme. (To be published. See Schlesinger 1949b.)

Finally, the demonstration by Henle and Rosenberg (1949) that multiplication of active Lee or PR8 virus can be completely inhibited by intra-allantoic inoculation, *during the constant period*, of large doses of homologous irradiated virus, also points away from the "receptor-block" hypothesis. In this case, the interfering virus exerts its effect even after the adsorption phase of the infectious cycle is past, the sudden access of large amounts of inactive virus to already infected cells interrupting the completion of the generative cycle within the cell. This observation more than any other supports the view that the irradiated virus, through the force of its mass, blocks or competes with the infecting virus for some cellular component(s?) essential not to the invasion by infecting particles, but to the completion of new infectious particles*. At what point this competition is effective is unknown. We do know from the work of Hoyle (1948) and Henle and Henle (1949) that the emergence of newly formed, fully infectious virus in the allantoic membrane is preceded by an increase in complement-fixing antigen and hemagglutinin. The effectiveness of homologous irradiated virus is greatest within the first hour after infection and then decreases progressively up to the end of the second third of the constant period. This suggests that inhibition of further viral development may occur at any stage of the intracellular cycle except that in which an increase in *complete* particles becomes detectable.

The suggestion that irradiated virus retains the capacity of exerting a specific effect within the cell, or at least at a step beyond the virus-receptor combination, is particularly intriguing because inoculation,

*It is of interest that the ability of capsular polysaccharides of Friedlander bacillus type B to inhibit the multiplication of mumps virus is believed to be due to blocking of some intracellular metabolic step, but not of the cellular receptors (Ginsberg, Goebel and Horsfall, 1948).

during the constant period, of irradiated *heterologous* virus *fails* to inhibit the completion of the growth cycle (Henle and Rosenberg 1949). This suggests that, in contrast to the homologous, the heterologous virus is *not* capable of invading cells already infected with the active virus and hence not in a position to block its complete cycle of reproduction. The analogy between this speculation and observations on phages is obvious: here the active virus already in the cell is no obstacle to the subsequent entrance of homologous particles, but it excludes the particles of the heterologous type ("mutual exclusion"). This is in good agreement with the fact that irradiated virus is equally effective in interfering with either homologous or heterologous active virus if inoculated before or simultaneously with the latter.

It is not unlikely that this blocking effect of homologous UV-inactivated virus may be related to the "auto-interfering" capacity of influenza virus in high concentration. Inoculation into eggs of dilute infected allantoic fluids yields virus of maximal titer. But if the seed consists of *undiluted* or concentrated allantoic fluid, the yield is lower (Henle and Henle, 1944a). When serial passages from egg to egg are done with undiluted PR8-infected allantoic fluids, the yield of hemagglutinating virus remains high in each passage with only a slight decrease, while the infective titer decreases progressively and markedly, especially in the third and fourth passage (von Magnus 1946, Gard and von Magnus 1946, Gard et al., 1947). The non-infective, hemagglutinating virus is believed to be a "precursor" of the fully developed virus. It is of special interest that continuation of the serial transfers beyond the fourth passage (when both, hemagglutinin and infective titer, are lowest) may lead to a rise in infective titer to maximal values in the fifth and a repetition of the shift thereafter (personal observation). Thus, the qualitative change from the fully developed, self-reproducing virus (sedimentation constant of about 700 S) into a modified form (sedimentation constant said to be about 500 S [Gard et al., 1947]) does not—as one might expect—end in the eventual disappearance of infectious particles altogether, but, on the contrary, in their sudden resurgence in full strength. The fact that hemagglutinin reaches maximal or almost maximal titers in each passage indicates that whatever interference takes place is not with *multiplication* of virus but only with its *completion.* This phenomenon has not as yet been adequately explained. It is important to realize, however, that it occurs only if the passage materials contain virus of either form in high concentration.

With so much speculation still required to bring order into the problem of interference between viruses as closely related to each other as

influenza types A and B, the situation involving viruses as dissimilar as influenza and W.E.E. appears even more confusing. At least it is clear from the results already mentioned that these viruses do not have common cellular receptors. The alternative explanation, i.e. common intra-cellular elements involved in the infectious process, has received some experimental support in recent observations in our laboratory. It had been found previously (Henle and Henle 1946, Vilches and Hirst 1947) that non-neurotropic strains of influenza virus, despite their "toxicity" and their interfering effect after intracerebral inoculation into mice, did not multiply in the mouse brain. Measurements of hemagglutination-inhibitor (receptor) titers of extracts from mouse brains harvested following intracerebral inoculation of the PR8, WS or Lee strains revealed progressive reduction in VHI with subsequent return to normal values (Schlesinger 1949b). The VHI decrease was of low order of magnitude when compared with that associated with infection with the neurotropic variant of the WS strain, but it was of the same order as that found in mouse lung extracts obtained from mice in the course of fatal infection with PR8 virus. This receptor decrease suggested some viral activity which was not in line with the observed decrease in infectious titer. It has now been possible to show that intracerebral inoculation of PR8-, WS-, or Lee-infected allantoic fluids in large amounts leads to the production, in mouse brain, of "incomplete" virus (hemagglutinin and complement-fixing antigen) in the face of progressive decrease in infective titer (Schlesinger 1950, details to be published). The production of hemagglutinin appears to be limited to a period corresponding to a single cycle of virus multiplication in the allantoic membrane. The maximum yield reached at the end of this period varies in direct proportion to the amount inoculated. This observation makes it plain that certain cells which are incapable of supporting the complete self-reproduction of these virus strains, nevertheless, allow the virus to undergo some phases of viral growth which, in fully susceptible cells, seem to precede the completion of the infectious particles. If the intracerebral inoculum is heavy enough, i.e. if a large number of cells in the brains are involved in this process, they are sufficiently impaired to lead to "toxic" death after 1 to 2 days. It appears reasonable to assume that it is these cells which are involved also in the infection with W.E.E. virus and with other neurotropic viruses with which influenza strains interfere. The necessity of using large doses of influenza virus to obtain significant degrees of interference is undoubtedly related to the necessity to infect many cells simultaneously because of the limitation of its incomplete growth to a single cycle.

In the light of the last mentioned and all the other recently accu-

mulated information on the "intermediate" stages in the multiplication of influenza virus, it is obviously of the greatest importance to re-evaluate the meaning and the definition of viral activity, in particular that of "inactivated" viruses which retain interfering ability.

The other big question mark is the identity of the links which the cell has to contribute to the viral reproduction. The problems have been well stated by Luria in his Essay on Virus Reproduction, and they are as essential to the problem of interference between animal-pathogenic viruses as to the general problem of viral multiplication.

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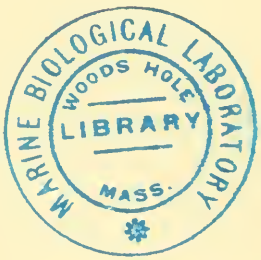

"MASKING," TRANSFORMATION, AND INTEREPIDEMIC SURVIVAL OF ANIMAL VIRUSES

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The opportunity of discussing some of the animal virus problems with a group of investigators skilled in the bacterial and plant virus fields is an extremely welcome one to me personally. In reading bacteriophage or plant virus papers, I have often found myself secretly wishing that our animal virus field could be reduced to the same simple and direct experimental approach frequently employed by phage and plant virus investigators.

There are several good and probably insurmountable reasons why this desirable state of affairs will never be achieved and why observations made with the bacterial viruses and the plant viruses will never be exactly and completely applicable to the animal virus field. For one thing the experimental hosts used by the bacteriophage and plant virus people have no circulating antibodies to foul up the efforts of researchers. While the immune response serves a very useful and practical purpose in the animal virus field, it does render impossible any strictly comparable *in vivo* comparisons between the behavior of animal viruses on the one hand and bacterial and plant viruses on the other. Of less importance, but still fairly serious in modifying the outcome of work with the animal viruses, are the roles played by cellular immunity, complications caused by unrecognized interfering infections, and the as yet poorly understood influence of the host's nutritional state on the course and outcome of animal virus infections. These are factors which play no or only a very minor role in work with the plant and bacterial viruses.

In pointing out these things, I am not trying to furnish an alibi for investigators of the animal viruses but merely endeavoring to indicate why some of our observations cannot be as beautifully clear cut as are many of those with bacteriophage or with certain of the plant viruses. I am very much inclined to agree with what Luria wrote in his statement concerning bacteriophage that the very fact that many of the gaps in our knowledge of viruses can be clearly visualized and delimited in phage analysis suggests that they may be filled more easily by work on bacteriophage than on other biological systems. Borrowing further from Luria's statement it is questionable how much light phage research can throw specifically on the events of other virus infections in that virus-host relationships may include systems so different that the findings with one will be only relatively indirectly applicable to the other. Bearing in mind the possibility that the com-

plications mentioned previously may nullify any useful comparison between the work with animal viruses that I propose to discuss and observations in the bacterial or plant virus field, I am going to present brief statements on the general subjects of "masking," transformation, and interepidemic survival of animal viruses and hope that they may initiate some mutually interesting discussion.

1. Virus "Masking"

For the sake of the present discussion, the term "masked" virus will be used to indicate a living virus which, for reasons that we do not fully understand, has been rendered noninfective and therefore not directly detectable by any of the tests for infectivity ordinarily used in demonstrating its presence. A "masked" virus is one which is known by circumstantial evidence or by a series of indirect tests to be present but which is not of itself directly demonstrable. If, in the case of the bacteriophage, the time gap between the disappearance of the initial virus and the appearance of the mature virus in an infected cell were a matter of days instead of minutes, the phage during this period might possibly be thought of as "masked" in the sense in which that term can be used in the animal virus field.

Two very good examples of "masking" of virus in the animal field and the two with which I have had the most personal experience are the cases of the papilloma virus as it exists in the tumors it causes in domestic rabbits and the swine influenza virus as it exists in its intermediate host, the swine lungworm. Since these two examples are fairly clear-cut and since each represents a phenomenon which may be of wide general importance, in the tumor field in the case of papilloma virus and in the broad field of epidemiology in the case of the swine influenza virus, they should furnish fit subject matter for this discussion.

a. "Masked" papilloma virus

I shall outline the case of the papilloma virus first. Briefly, the situation is as follows: Cottontail rabbits in our Middle Western States have a disease characterized by the occurrence of papillomas (warts) over various parts of their bodies. These warty growths are rich in a virus which when applied to the scarified skin of other cottontail rabbits or of domestic rabbits induces the appearance of papillomas apparently identical to those seen on the naturally infected wild cottontails (1). The papilloma virus is usually readily transmissible in series through cottontail rabbits, and ordinarily one has no difficulty in passing the agent indefinitely from cottontail to cottontail. In this species therefore

the papilloma has all of the earmarks of a virus-induced tumor and is analogous in this respect to the chicken tumors of the Rous type. No investigator would seriously doubt causal relationship of the papilloma virus to the epithelial tumors which follow its inoculation into cottontail rabbits.

The case of the domestic rabbit, however, presents quite another set of circumstances. As mentioned previously, if papilloma virus of cottontail rabbit origin is applied to the scarified skin of domestic rabbits, papillomas result just as they do in the wild rabbit and their general appearance is the same. However, if one attempts to transmit the virus serially beyond the first domestic rabbit passage, one makes the rather startling discovery that the domestic rabbit papillomas contain no virus demonstrable by direct means. Rabbits, either cottontail or domestic, inoculated in the usual fashion with suspensions of domestic rabbit papillomas fail to develop papillomas or exhibit any evidence of infection. The situation in the domestic rabbit is therefore a very puzzling one. The evidence that the domestic rabbit papilloma has a virus as its cause is about as direct as could be hoped for — the wild rabbit virus was placed on the scarified domestic rabbit skin and the tumors developed at the sites where the virus was deposited. However, if an investigator unfamiliar with the method used in inducing the warts were presented with a domestic rabbit suffering full blown papillomatosis and were told to determine the etiology of the condition, he would be completely unable to demonstrate that it had a viral cause. He would almost certainly characterize the tumor as a mammalian neoplasm of nonviral etiology. As such, the condition can be transmitted in series to other domestic rabbits by tumor grafting in a manner similar to that employed in working with nonviral mammalian tumors.

Thus with a single clinical entity (papillomatosis), in two species of animals, we have examples of a condition which in one species, the cottontail, is a typical readily transmissible virus tumor and in the other species, the domestic rabbit, is apparently a nonviral tumor typical of this class of mammalian neoplasms. Fortunately, for the sake of completeness of the scientific record and with wild rabbit virus available as a test reagent, there are indirect means of demonstrating that papilloma virus is present in the domestic rabbit tumors even when these become malignant and progress to cancer. These methods are immunological and, briefly, make use of the virus neutralization test or the demonstration of active immunity. Thus a domestic rabbit afflicted with papillomatosis will have antibodies in its blood serum capable of completely neutralizing papilloma virus of cottontail origin. Such a

rabbit will also be refractory to reinfection with fresh cottontail papilloma virus. Another bit of indirect evidence indicating the persistence of papilloma virus in domestic rabbit papillomas is furnished by the observation that a noninfective suspension of domestic rabbit papilloma tissue, if injected subcutaneously or intraperitoneally into another domestic rabbit, will render that animal immune to cottontail papilloma virus as indicated by the development of virus neutralizing antibodies in the blood serum and a refractory state to active infection with the virus (2). The evidence therefore is that even though papilloma virus in an infective form is not present in domestic rabbit tumors, it is present in an occult or "masked" form which is still capable of eliciting the characteristic immune responses of fully infective papilloma virus.

The mechanism by which papilloma virus is "masked" in the domestic rabbit tumors is not known. Some investigators believe that virus-neutralizing antibody constitutes the "masking" agent. This scarcely seems the correct explanation because virus-neutralizing antibody is present in the serum of infected cottontail rabbits without "masking" of the cottontail papilloma virus.

Aside from serving as a good example of a "masked" virus, papilloma virus as it exists in domestic rabbit tumors points to the possibility that this class of agents ("masked" viruses) may have wide implications in the tumor field in general and may play a large role in that group of mammalian tumors now believed to be nonviral in causation. It is probably farfetched to think that very many apparently spontaneous and noninfectious mammalian tumors have as their cause a virus native to another species, but the case of the papilloma virus indicates that such an eventuality is not beyond the realm of possibility. Less farfetched is the possibility that, in some tumors in which a carcinogenic virus cannot be demonstrated by any means yet tried, virus "masking" after the manner of the papilloma agent may be responsible for the apparent absence of virus. It seems likely that all of the carcinogenic viruses amenable to conventional techniques of isolation have been brought to light. Yet the very existence in a tumor of an agent like "masked" papilloma virus indicates the possibility that agents more elusive than the conventional tumor viruses may exist and may even be quite common so far as anything we know. The possibility that "masked" papilloma virus may represent the prototype of these more elusive carcinogenic agents makes it especially urgent that we learn as promptly as possible just what constitutes the "masking" process in the case of the papilloma virus. Thus far we do not even have a half promising lead.

b. "Masked" swine influenza virus

The second example of "masked" virus that I am going to outline for this discussion is that of the swine influenza virus in its lungworm intermediate host. Actually this example of "masking" plays a large role in the second topic that I am to present for discussion at this conference, namely, the interepidemic survival of animal viruses. As a consequence this discussion of "masked" influenza virus in the swine lungworm is a natural transition between the two topics.

The epidemiology of swine influenza is quite simple once an epizootic has started. As most of you probably know, this disease has a complex etiology being caused by infection with a bacterium, *Hemophilus influenza suis*, and the swine influenza virus acting in concert (3). The disease itself is a highly contagious respiratory infection and the spread from sick to well is rapid and extensive. Once a case has appeared on a farm or in a community, orthodox epidemiological considerations are all that are required to explain its widespread dissemination. However, getting that first case of swine influenza started from "scratch," so to speak, is a stickler. I might point out in passing that the problem of accounting for the first case in an epidemic is not unique to the case of swine influenza, but is shared by most, if not all, of the other epidemic diseases of man or animals.

Characteristically swine influenza epizootics are of annual occurrence in the Middle West and begin explosively usually the last week in October or the first week in November (4). The build up of cases is extremely rapid and one gains the impression that either the disease spreads with unbelievable rapidity or that it has arisen at many different foci simultaneously. After the initial widespread outbreak, fresh swine droves are infected in smaller and smaller numbers until by late December or early January, as a rule, the epizootic appears to have run its course and swine influenza disappears as a farm infection until the following October or November. Consideration of the epidemiological situation made it appear quite obvious either that the causative agents arose *de novo* each year or that a reservoir host mechanism existed which was capable of preserving the agents throughout the nine months of the year that the disease is not active. Persistence of the bacterial component *Hemophilus influenzae suis* for long periods of time in the respiratory tracts of recovered animals can be demonstrated. However, similar persistence of the swine influenza virus cannot be shown and ordinarily it disappears from the respiratory tracts of recovered animals on about the eighth day after their initial infection.

As it turns out, there is an intermediate host capable of preserving the swine influenza virus in nature for long periods of time. This inter-

mediate host is the swine lungworm. Since the lungworm cycle is a rather complicated one, it probably should be outlined briefly at this point for the sake of clarity (5).

The lungworm is a nematode parasitic in the bronchioles of the bases of the lungs of swine. The female lungworm lays fully embryonated eggs in the bronchi of the swine she infests. These are coughed up, swallowed, and reach the outer world in the feces. Their further development then is dependent upon their being ingested by an earthworm. Once within the earthworm, the lungworm eggs hatch and the larvæ develop to the third or infective stage, usually localizing in the calciferous glands and hearts of the parasitized earthworm. They persist in this stage until the earthworm is eaten by a pig. In the pig the lungworms undergo two further developmental stages finally reaching the respiratory tract, by way of the blood stream and lymphatics, where they become adults. The whole of this cycle can occupy a span of several years for its completion, or, under the most favorable conditions, can be completed in a little more than a month.

In the above cycle the larvæ developing from lungworm ova laid during the time the host pig is undergoing an attack of swine influenza, or even from those laid for at least a short period after recovery, will be carriers of swine influenza virus (6). A most puzzling feature of this situation, however, is that the virus cannot be detected by direct means either in the larvæ in their intermediate hosts or in the adult lungworms after transmission to their definitive host, the pig. It appears to be present in an occult or "masked" form and evidence of its presence is furnished only by its subsequent behavior under very specialized conditions. Swine that have become parasitized with lungworms known by the subsequent course of events to be carriers of "masked" virus do not come down directly with swine influenza. Instead they remain to all outward appearances perfectly normal pigs. However, actually they are in a very precarious situation so far as their eventual well-being is concerned because all that is required to bring them down with a severe or even fatal influenza infection is the application of some stimulus, of itself completely harmless. Several such provocative stimuli have been used, but the one that has proven most regularly effective consists in the administration of multiple injections of the bacterium, *H. influenzae suis*. I should add that the provocation of "masked" influenza virus succeeds under experimental conditions only in the period between September and April. It has uniformly failed in the months of May, June, July, and August.

The nature of the "masking" process of swine influenza virus in the lungworm is not at all known and even the indirect means which have

been successful in demonstrating the presence of "masked" papilloma virus in domestic rabbit tumors have failed to shed light in the case of the influenza virus in its lungworm intermediate host. Thus far "unmasking" of the virus has been effected only by the rather complicated procedure of transmitting the infected lungworms to susceptible swine and there provoking the virus to activity *in vivo*. Fully infective virus is detectable only at either end of the procedure. Stanley, a long time ago, compared the phenomenon very aptly with that of a train passing through a tunnel: One can see the train as it enters and as it leaves, but it is no more apparent while in the tunnel than is swine influenza virus while in its intermediate host. Incidentally this same tunnel simile could be just as well applied in the case of bacteriophage infections of susceptible bacteria in which the initial virus disappears only to reappear later as the mature virus.

2. *Interepidemic Survival of Animal Viruses*

a. Swine influenza

The preceding account of the "masking" of swine influenza virus in its lungworm intermediate host fairly well explains the mechanism by which swine influenza virus survives in nature from one outbreak to the next. Field experiments have shown that the phenomenon is not an isolated laboratory trick, but actually does take place on a large scale in nature. Lungworm larvae from earthworms dug in the pig pastures on numerous Midwestern farms where influenza is of annual occurrence have been shown experimentally to be carriers of "masked" influenza virus (7).

b. Salmon poisoning

Salmon poisoning in dogs is another infectious disease of probable viral origin in which the causative virus hides out in a reservoir worm host between its appearances as the cause of sporadic disease in dogs (8). In this case, however, the worm is a trematode having a fish and a snail as intermediates in its life cycle. Here again the virus appears to be "masked" during at least a part of the cycle in its intermediate host since it has never been demonstrated by direct means during the part of its life history that it spends in the snail.

c. Bovine pseudorabies

Bovine pseudorabies, a virus disease of cattle, tides over from one appearance to the next in a rather interesting fashion. The disease caused in cattle by this virus is not contagious, and so far as anyone knows, never transmits directly from one sick cow to a normal one. The

disease caused by the pseudorabies virus is uniformly fatal and kills all cattle it infects usually within forty-eight hours of the onset of symptoms. It is evident therefore that unless some extra-bovine host were involved in the epidemiology of the disease, pseudorabies virus should have died out and been forever lost in the first cow it infected. Fortunately for the virus, an intermediate host, capable of perpetuating it in nature and of getting it transmitted from cow to cow, is available. This intermediate and reservoir host is the hog (9). In swine, pseudorabies virus is a contagious disease transmitting readily from pig to pig and persisting in infected swine for upwards of two weeks. Unlike pseudorabies in cattle, swine infections are extremely mild. In fact, they are so mild that, though the virus is known to cause extensive infections in Middle Western swine, these infections are seldom recognized clinically. Porcine pseudorabies might therefore be termed a silent epizootic disease and its presence on Middle Western farms is not recognized unless the causative virus escapes from its porcine host and spreads to cattle. Pseudorabies virus is present in and on the noses of infected swine in relatively high concentration and is transmitted from this site to cattle through abrasions on the skin. The epidemiological situation in pseudorabies is somewhat reminiscent of that prevailing in the case of lysogenic cultures of bacteria. If one were to translate the pseudorabies epidemiology into terms of bacteriophagy, the swine in the picture would become the lysogenic carrier strain and the cattle would be analogous to the indicator organisms. The immunity reaction of swine to the pseudorabies virus enters to spoil the complete parallelism of the two pictures. As I mentioned above, pseudorabies virus persists in the noses of infected swine for upwards of two weeks. It disappears then because by this time the pig has become immunized and either frees itself of virus or renders its virus noninfectious. Lysogenic bacterial carrier strains do not have an immunity response to contend with and may therefore remain lysogenic indefinitely.

d. East African swine fever

East African swine fever is another virus infection in which the responsible intermediate host plays a role somewhat analogous to a lysogenic organism in spreading its infection from one recognized outbreak to the next. In this case the wart hog is the healthy carrier of a virus which it transmits periodically to domestic swine with devastatingly fatal consequences to the domestic pigs (10).

e. Snotsiekte

Another similar example which may be mentioned is that of Snotsiekte in African cattle (11). In this instance the gnu or wildebeeste

would constitute the analogue of the lysogenic carrier strain in being the healthy carrier of Snotsiekte virus which it transmits by contact as a serious disease-producing agent to domestic cattle, the analogue of the indicator organism.

There are lots of other viruses which survive from one periodic outbreak to the next, but unfortunately our knowledge of how they survive and where they hide out during the time that they are not causing such diseases as hog cholera, poliomyelitis, measles, cattle plague, foot-and-mouth disease, etc., is not as complete as in the instances that I have just outlined.

The examples that were cited above embody only two mechanisms for interepidemic survival, namely, persistence of the causative virus in a worm intermediate host and persistence of the virus in a species in which it causes either no or only mild symptoms. Both of these mechanisms are admirably suited to insure the prolonged survival of viruses and to perpetuate the diseases they cause from the period of mass immunization at the end of one epidemic to the period of mass susceptibility signaling the onset of the next epidemic. It would be strange if nature abandoned these mechanisms for perpetuating virus infections after only a few applications, and it would seem quite reasonable that some, if not many, of the viruses with whose whereabouts between epidemics we are not as yet familiar might fall within one or the other of the two mechanisms outlined.

3. *Transformation of Viruses*

Though frequently suspected of taking place under natural conditions, there appears to be but one experimentally reproducible example of transformation in the animal virus field. This is the changing of rabbit fibroma virus to that of infectious myxoma — the Berry-Dedrick phenomenon. This phenomenon appears superficially to be something of a mixture of "recombination" and "multiplicity reactivation" as these terms are applied in the bacteriophage field and should therefore be a good subject to present for discussion at this symposium. Since some of you may not be completely familiar with the background of the Berry-Dedrick transformation, I shall review it briefly.

The two viruses involved in the reaction are the myxoma and the fibroma viruses, both of rabbit origin. The myxoma virus appeared for the first time in Brazil and has not occurred as a cause of natural disease in domestic rabbits outside of that country except for its introduction from there into California rabbitries. The disease that this virus causes in domestic rabbits is highly fatal and readily contagious.

The fibroma virus was first isolated from a tumor in a cottontail rabbit shot near Princeton Junction, New Jersey. Sporadic cases of fibroma virus infection have since been observed in various scattered regions of New Jersey, in northern New York State, and in some areas of the Middle West. It is not a commonly recognized condition in wild rabbits. The virus is readily transmissible by inoculation to both cottontail and domestic rabbits but is not contagious in either species. The condition is never fatal and causes only benign fibromatous tumors at sites of inoculation in either species.

From the above facts it appeared that the two viruses and the diseases they caused were quite different and distinct; myxoma, a disease apparently native to domestic rabbits was contagious and regularly fatal, while fibroma, a disease seemingly native to cottontail rabbits, was not contagious and never fatal. The two conditions did have one point of similarity, however, and this was the frequent superficial resemblance of the initial lesion, developing at the site of inoculation with myxoma virus, to the early fibromas developing at the sites of inoculation with fibroma virus. It was this similarity between the initial local lesions of the two conditions that early led to an immunological comparison of the two viruses and the discovery that infection of rabbits with fibroma virus rendered them immune to fibroma and unusually refractory to myxoma. Rabbits that had recovered from fibromatosis and that were subsequently inoculated with myxoma virus came down with a myxomatosis showing less manifestations of generalization than usual and furthermore such animals almost never died (12). It appeared from this that a previous fibroma virus infection, though not immunizing rabbits against myxoma virus, did give sufficient protection to prevent a fatal outcome. This state of increased resistance to myxoma virus induced by fibroma infection began very early and at some time between the second and fourth day was frequently demonstrable. At no time, however, did antibodies capable of neutralizing myxoma virus appear in the blood serum of fibroma recovered animals, though fibroma virus-neutralizing antibodies were readily demonstrable. The immune response in the reverse direction was more complete and the very rare rabbit that survived a primary myxoma virus infection was not only resistant to infection both with the myxoma and the fibroma viruses, but also possessed antibodies in its blood serum that were capable of neutralizing both viruses.

The immunological and serological data obtained in the cross-protection studies outlined briefly above indicated that the myxoma virus contained antigenic components essential to the production of complete fibroma virus immunity. On this basis, the incomplete protection of

rabbits against myxoma virus conferred by infection with fibroma virus was interpreted as indicating that the fibroma virus was antigenically only a partial replica of myxoma virus. The antigenic components comprising fibroma virus and common also to the myxoma virus were sufficient to establish in fibroma-infected rabbits a state of resistance to myxoma, but, because they represented only partially the antigenic composition of myxoma virus, this resistance was not the complete immunity conferred reciprocally by two identical viruses. In short, the cross-immunological data suggested strongly that myxoma virus was composed of fibroma virus plus some other antigenic component and that this other hypothetical component was responsible for the lethal virulence of myxoma virus in rabbits.

The possibility that myxoma virus might be a complex containing fibroma virus as one of its components aroused interest in two laboratories, but the workers in these laboratories approached the problem from exactly opposite directions. In one instance attempts were made to convert myxoma to fibroma virus by removing the hypothetical component of the myxoma agent, while in the other the conversion of fibroma to myxoma virus was attempted by adding the hypothetical component of myxoma virus to fibroma virus.

In the case of the first experimental approach, myxoma virus was passed through fibroma-recovered rabbits for eight serial passages testing virus at each passage for its possible reversion to the fibroma type (13). No alteration in the original fully lethal character of the myxoma virus was shown. In another group of experiments designed to achieve the same end, myxoma virus was passed in series through cottontail rabbits for ten passages covering a total elapsed time of 140 days during which the myxoma virus was not out of cottontail tissue (14). Myxoma virus, as I neglected to mention earlier, induces in cottontails a nonfatal disease in which the only manifestation of infection is a fibromatous tumor, identical in appearance to naturally occurring cottontail fibromas, developing at the site of inoculation. It was reasoned that, since myxoma virus in the cottontail induced lesions and a clinical picture similar to that caused in this species by fibroma virus, some change in myxoma virus might be expected to occur as a result of its prolonged sojourn in cottontails. However, such did not prove to be the case and the virus recovered from the cottontail lesions even at the end of ten serial passages and a total residence of 140 days was still fully lethally pathogenic myxoma virus as demonstrated by its activity in domestic rabbits. It was apparent that, at least so far as the techniques applied in these experiments were concerned, no removal of a hypo-

thetical component from myxoma virus to convert it to fibroma virus had been achieved.

The other approach, namely, the experiments of Berry and Dedrick (15), in which an effort was made to transform fibroma virus to myxoma virus by the addition of a hypothetical component from myxoma virus, turned out successfully. The apparent close immunological relationship between the fibroma and myxoma viruses had led Berry and Dedrick to explore this hypothesis and their approach to the problem had been suggested by Griffith's studies of transformation of pneumococcal types. Briefly, they found that, if myxoma virus which had been inactivated by heating at either 60°C. or 75°C. was mixed with live fibroma virus and administered to domestic rabbits, the inoculated animals, instead of developing the mild fibroma, frequently came down with fatal myxomatosis. Control rabbits receiving heated myxoma virus alone, of course, remained normal. These experiments suggested that some component of myxoma virus, not destroyed at 75°C., was capable of combining with fibroma virus to convert that agent to myxoma virus.

Although Berry and Dedrick published no detailed account of their work, several investigators including myself have repeated it in essence and duplicated their observation. Hurst has published the most extensive account of the phenomenon, and it appears from his work that the proportions of heated myxoma to living fibroma virus are of considerable importance in achieving the transformation (16). Hurst's experiments indicate that, using 10 per cent infected tissue as a source of either myxoma or fibroma virus, a ratio of 25 or 50 parts of heated myxoma virus to 1 part of live fibroma virus most regularly results in transformation. If the proportion of live fibroma to heated myxoma virus in the mixture is too great, transformation fails to occur and the inoculated rabbits develop only fibroma. It is believed that these failures of transformation may result from too ready an immunological response to fibroma virus infection. As mentioned earlier, fibroma virus induces a state of increased resistance to myxoma virus which may become evident in as short a period as 2 to 4 days. If therefore the proportion of fibroma virus in the transformation mixture is too great, the reaction of resistance engendered by the fibroma virus prevents the appearance or detection of any small amounts of myxoma virus that may be produced and the end result will be apparent failure of transformation. It seems quite apparent that the transformation of fibroma to myxoma virus is a delicately balanced *in vivo* reaction depending at least partially for its success on the correct timing of the transformation in relation to the establishment of untransformed fibroma virus in the host. Almost certainly the ratio of transformed myxoma virus units to un-

transformed fibroma virus units has to be at a certain critical level at the time that the fibroma virus would normally begin exerting its protective effect within the host for the transformation to become apparent. It is also probable that other factors than the mere ratio of fibroma to heated myxoma virus are of importance in determining the success or failure of transformation because Dr. Margaret Smith, working with the phenomenon in my laboratory, frequently experienced failures even when all of the known factors were controlled to the letter.

The exact mechanism by which heat-inactivated myxoma virus transforms fibroma virus to myxoma virus is not understood. I mentioned at the outset that the phenomenon appears superficially to be something of a mixture of "recombination" and "multiplicity reactivation." The recombination phase of the affair concerns mainly the fibroma virus which is the living component of the reaction mixture. To completely parallel the phage phenomenon of recombination, it should acquire new characters from combination with living myxoma virus. Actually the component of myxoma virus with which fibroma virus apparently combines in the transformation is capable of being perpetuated as such once it combines with fibroma virus. It thus seems potentially at least to possess one of the attributes thought to be characteristic of living matter, namely, reproduction. The "multiplicity reactivation" phase of the myxoma transformation concerns mainly the thermo-stable component of myxoma virus since alone this component has all of the earmarks of an inactivated material so far as propagation "on its own" is concerned. However, in combination with fibroma virus this apparently inactivated and "dead" component becomes reactivated apparently as a result of the replacement of the component, fibroma virus, which was destroyed during the heat-inactivation process.

From all of this it seems to me that the Berry-Dedrick phenomenon of myxoma virus transformation has quite a lot in common with some of the exciting tricks that bacteriophages can be made to do. The complicating factor, as I mentioned at the outset, which prevents and will continue to prevent any absolute parallelism between observations made with the animal viruses on the one hand and with the bacterial and plant viruses on the other is the presence, in the hosts of the animal viruses, of antiviral immunity. This specific immune response to infecting viruses will forever be present in the animal field to interfere with or prevent those beautifully clear-cut and definite results so often achieved by the plant virus and bacteriophage people. However, despite this, there is undoubtedly much that can be learned from the plant and bacterial virus work that can be rather directly applied with profit in the animal virus field.

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IMMUNOLOGICAL PROPERTIES OF PLANT VIRUSES[1]

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Serological reactions — It is now well established that plant viruses are antigenic and evidence obtained from many studies, particularly those dealing with purified virus preparations, strongly favors the view that the specific antigens found in virus-affected plants are the viruses themselves, rather than products of the host plant produced as a result of virus infection. Most of the evidence of the antigenicity of plant viruses has been obtained from precipitin reaction and complement fixation tests. With these techniques the results appear to be clear cut especially in the case of the more stable viruses that exist in host plants in relatively high concentrations. Certain plant viruses, which have not given antigenic reactions when crude or clarified sap of affected plants was used, have been shown to be antigenic when purification or concentration procedures were followed.

Studies of anaphylactic reactions have been quite meager and those reported have dealt chiefly with the tobacco mosaic virus. The conclusion drawn from this work is that purified tobacco mosaic virus induces anaphylactic shock "in vivo" although it is much less anaphylactogenic than animal proteins.

A fourth test of antigenicity of plant viruses and one which at first sight would seem to be the simplest means of demonstrating serological activity of plant viruses is that of neutralization of infectivity. However, it has been found that both normal and heterologous antisera inhibit infectivity. Thus accurate tests and adequate controls are necessary to measure the specific effect of homologous sera in reducing infectivity. From the reported studies on neutralization of infectivity the following facts seem to be established:

A. Normal sera and heterologous sera have a non-specific effect in

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[1]For more detailed discussion of this subject and for specific literature citations the reader is referred to the following:

Bawden, F. C. Plant viruses and virus diseases. *Chronica Botanica Co.*, Waltham, Mass., U. S. A. 1943.

Price, W. C. Acquired immunity from plant virus diseases. *Quart. Rev. of Biology*, 15:3. 1940.

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reducing infectivity of plant viruses. Non-specific reduction takes place immediately upon mixing normal antiserum and antigen.

B. Each antiserum has a specific effect in reducing the infectivity of the virus used in making that antiserum. Specific effect increases with time.

C. Specific reduction in infectivity results from the action of antiserum on the virus and not on the host.

D. The virus is neutralized rather than inactivated since some infectivity is regained when neutralized mixtures of virus and antiserum are either diluted or incubated with pepsin.

Serological reactions as an aid in identification of viruses — The specificity of serological reactions permits their use in the identification of viruses. However, inasmuch as these reactions are only group specific, identification by this means is not exact. Different strains of the same virus usually react similarly when tested against one another in straight serological tests. Reaction between the virus of one strain against the antisera of another strain, however, does not prove that these strains are identical antigenically, but shows merely that the two strains contain certain common antigens. On the basis of cross-absorption procedures, serologic tests with certain related virus strains suggest that each virus is not a simple unit antigen but that each may carry a number of different determinant groups which will likewise be contained in the antisera of each virus.

It has been observed that the range of antigen dilution over which precipitation occurs may vary widely when two related virus strains are tested against a given antiserum.

The lack of sufficient antigen in expressed sap is no doubt sometimes responsible for failures to obtain precipitin reactions. In fact, there are recorded instances where concentration by precipitation or by high-speed centrifugation have established strain relationships that were not detected when crude sap was used.

With an understanding of the phenomena listed above and their use in serological technique, it seems that these methods may be not only of value in grouping virus strains but that they may also become useful in indicating the degree of relationship between different strains. From the standpoint of virus classification it appears that viruses must be arranged in larger groupings or families on the basis of properties or characteristics other than serological responses, but the fact that

these immunologic reactions are of value in determining strain relationships is an important advance.

Cross-protection — Cross-protection or strain interference has been widely used in the identification of viruses. Because of the rather general agreement between cross-protection and serologic identifications of viruses, the former should be mentioned at least briefly here.

Cross-protection involves two general types of behavior in virus-affected plants. The first and most common is the protection afforded plants against virulent strains of virus by a previous infection with avirulent strains. It is perhaps sufficient to state that this phenomenon is encountered quite often in the plant virus field and that it has been of value in determining strain relationships. In general, strain identification by serologic and cross-protection methods have been in accord, but at least in a few reported cases the results have not been in agreement. Recent work with certain viruses that in earlier studies had not shown the same relationship by the two methods has demonstrated that disagreement resulted from the lack of a sufficiently sensitive serological technique.

The degree of protection afforded plants by one virus strain against a second is influenced by numerous factors, — age of plant species, time and method of infection, etc. Standardizing both serologic and cross-protection techniques may prove successful for determining the antigenic relationships of other viruses that have shown relationships by only the cross-protection test.

Although it is generally true that plants previously invaded by an avirulent strain of virus are protected against a virulent strain of the same virus, there is at least one striking exception in the case of the virus causing the curly top disease of sugar beets. This virus consists of numerous strains which are without question closely related. In this case it has been well established that in sugar beets avirulent strains do not protect against virulent strains. Virulent strains superimposed on avirulent strains induce infection and the host plant then shows symptoms of the virulent strain. Furthermore, plants already infected with virulent strains are not protected from infection with avirulent strains. The symptoms of the latter are of course masked, but the virus becomes established in the plant and can be recovered separately from the plants having mixed infections.[2]

The second type of behavior of virus-infected plants that has been

[2]Giddings, N. J. Some interrelationships of virus strains in sugar beet curly top. *Phytopath.*, 40:4, 377-388. 1950.

studied in relation to cross-protection is that commonly referred to as "recovery." With some viruses, infected plants gradually pass from an acute or severe symptom stage to a chronic, mildly affected condition. In the latter stage the infected plants may at times show no symptoms although the virus is present in them. Such recovered plants generally manifest no additional symptoms when reinoculated with the same or related virus strains.

4. *Acquired immunity*

The two types of behavior mentioned above under the section on "cross-protection" have been described by some workers as "acquired immunity." The use of the term "acquired immunity" for these reactions has been vigorously criticized by many workers. Certainly these reactions differ widely from acquired immunity as known in the field of animal immunology. Strong arguments have been advanced that these plant reactions are not immunological.

Incomplete recovery, persistence of the virus in recovered plants, and the lack of a circulatory system in plants comparable to that of animals are the chief arguments advanced that the acquired resistance found in plants that recover from severe symptom stages of virus infection is not immunological. It has been suggested that the mild or chronic symptoms are merely a second stage of symptomatology that follows after the viruses reach the growing point and are able to invade the tissues uniformly in the early stages of differentiation.

The behavior of plants that recover from the curly top virus seems to differ from other reported studies and more nearly approaches true immunological reactions.[3] Briefly these reactions are as follows:

A. Turkish tobacco plants commonly recover from severe symptoms of curly top and are resistant to reinoculation.

B. Commercial varieties of tomato do not recover.

C. Recovered tobacco plants contain virus unchanged in virulence.

D. Healthy tobacco plants infected by grafting with recovered tobacco plants develop mild symptoms.

E. Healthy tomato plans develop severe symptoms when infected by means of insect transfer from recovered tobacco, but develop mild symptoms when infected by grafting with recovered tobacco.

F. The mildly-affected tomato plants contain virulent virus.

[3]Wallace, James M. Acquired immunity from curly top in tobacco and tomato. *Jour. Agr. Res.*, 69:5, 187-214. 1944.

G. The mildly-affected tomato plants carry a certain degree of protection from additional infections.

H. The "acquired immunity" of tomato plants is retained through many vegetative generations.

I. The degree of protection in tomato plants varies with different strains of the virus.

J. Studies with 12 strains of the virus showed that plants immunized against one strain protected against certain strains but not others. The degree of protection as shown by cross-inoculation ranged from very slight to complete.

The information presented above is sufficient to show that in the case of curly top the responses differ from the usual plant-virus recovery phenomena. The reactions in this instance closely parallel certain known immunologic processes in animals. The analogy may be expressed simply as follows:

a. "Active immunity" initiated in tobacco plants.

b. "Passive immunization" of tomato plants that do not acquire immunity actively.

c. Strain specificity.

In comparing these plant responses with known immunologic reactions in animals it is at least of interest to point out that the curly top virus is known to be limited largely, if not entirely, to the phloem sieve tubes which carry the elaborated plant food. Thus, a uniform medium is provided for the virus at all times. Such a virus-tissue relationship in plants would, it seems, afford conditions somewhat similar to those under which antibodies are produced in animals.

It has been suggested by some virus investigators that these so-called immunologic reactions of curly-top-affected plants result from the use of undetected virus-strain mixtures. This presupposes (1) that the beet leafhopper, the vector of the virus, can obtain avirulent strain A and virulent strain B by feeding on a diseased sugar beet for example, (2) that the vector can introduce the two strains into a tobacco plant, and (3) that non-viruliferous vectors can then acquire only virulent strain B even though strain A is present in the tobacco plant in sufficient quantity to protect other tobacco and tomato plants infected by graft transfer.

Such an explanation is completely unacceptable to workers experienced with the curly top virus strains and vector relationships. Over a period of many years, repeated passage of the established virus strains

through numerous different plant hosts, involving both vector and graft transfer, has revealed no evidence of strain mixtures. Furthermore the vectors pick up two or more strains of virus from known mixtures and when used for inoculation of plants in repeated, short-time feedings may infect those plants with individual strains or with various combinations of the strains carried.[4]

There is much experimental evidence against the suggestion that the behavior of the plants in this instance results from strain interference. If it is accepted that the vector, by selection, acquires only the virulent strain from the immunized tobacco or tomato plants then it must be agreed that the vector screens out the protecting strain. The facts are that healthy tobacco plants infected by insect transfer of the virus from immunized plants of either tobacco or tomato repeat the process of recovery and again confer protection to tomato plants that are infected by graft transfer from the tobacco. Thus it seems that if these phenomena are to be explained on the basis of strain interference it is necessary to accept firstly, that the vectors are capable of strain selectivity when there is much experimental evidence that such is not the case, and secondly, that when any single strain is introduced into a tobacco plant a second strain always arises by some process which confers protection to tomato plants graft-infected from that plant but which cannot be transferred by the insect vectors.

Recovery of plants from the symptoms of virus diseases, that is, the gradual passage of the plants from a severe stage to a chronically mild or symptomless stage, and their resistance to reinfection thereafter could arise it seems by non-immunologic processes. Up to that same point the recovery of tobacco plants from curly top provides no good evidence of immunologic responses. However, the passage of the acquired condition of tolerance of the virus and resistance to reinoculation in recovered tobacco plants to tomato plants which very rarely initiate the recovery reaction, and the further transfer of these properties from tomato to tomato indicate that these responses differ from other reported instances of recovery from plant viruses. The question still remains as to whether or not protective or antibody-like substances are involved in these phenomena.

COMMENT

S. E. Luria

Dr. Wallace's results on transmission of curly-top infection in tobacco and tomato (mild disease by graft transmission, severe disease

[4]See footnote 2.

by leafhopper transmission) may be interpreted in terms of interference between a virulent and an avirulent strain if we postulate, not that the virus is a "mixture" of two different, independent strains, but that there are two mutant forms of virus in equilibrium in recovered plants, because of mutations in both directions, of selection pressures, or both. Recovery would correspond to the condition in which the mild virus predominates, although both viruses are present. Graft transmission from a recovered plant would give a mild disease because of massive transmission of a virus population already in equilibrium, with an opportunity for the mild mutant to prevent a quick spread of the virulent one. We should suppose that the insect transfers the severe disease, not necessarily because it selects the virulent virus, but because it transmits it in such a way that its spread is not checked by concurrently inoculated mild virus. This in turn could be due to selective growth of the virulent virus in the insect, or to transmission of small virus amounts with chances for one virus type only to be introduced in each cell or group of cells, or to lower ability of the mild virus to spread if inoculated in small amounts. In tomatoes, which recover less frequently, the severe virus may be less easily overcome by the mild mutant because of any one of a number of conceivable possibilities.

This suggestion is not intended to "explain" the results, but only to point out how genetic considerations may be useful in suggesting alternative interpretations of complex cases in virus-host relationship.

A SYLLABUS ON PROCEDURES, FACTS, AND INTERPRETATIONS IN PHAGE

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1. . . . . Introduction
2. . . . *I. Free Phage*
3. . . . . Identification of infective units with physical particles.
4. . . . . Morphology.
   (a) Electron micrographs.
   (b) Osmotic shock.
5. . . . . Chemical composition of phages.
6. . . . . Stability.
7. . . . . Serology.
   (a) Bacteriophages as antigens.
   (b) Phage anti-phage reaction.
8. . . . . Virus mutants.
9. . . . . Taxonomy.
10. . . . . Cofactor activations.
11. . . . . Radiation inactivation.
12. . . . *II. Glossary*
13. . . . *III. Adsorption*
14. . . . . Adsorption without killing of the bacteria
15. . . . . Mechanism of the adsorption process.
16. . . . . The bacterial change from sensitivity to resistance.
17. . . . . Receptor spots.
17a. . . . . Supplementary information on receptor spots, by *W. Weidel*
18. . . . *IV. Invasion*
19. . . . . Characteristics of invasion.
20. . . . . Phage T5 in the absence of calcium.
21. . . . . Ultra-violet treated phage.
V. Multiplication
22. . . . . Burst size distribution—correlation with size of bacteria.
23. . . . . Latent period.
24. . . . . Influence of growth medium.
25. . . . . Dependence of the latent period on the temperature.
26. . . . . Dependence of growth on specific supplies.
27. . . . . Premature lysis.
28. . . . . Fate of the infecting particle.
VI. Mixed Infections
29. . . . . With particles differing by one mutational step.

30.....With mutants carrying two genetic markers. Recombination. Linkage.
31.....With unrelated strains. Interference and mutual exclusion.
32.....With related strains. (T_2, T_4, T6)

VII. Reactivation Phenomena

33.....Photoreactivation.
34.....Multiplicity reactivation.
35.....*VIII. UV Method for Following Early Stages of Intracellular Development*
36....*IX. Cytology*
37....*X. Lysogenesis*
38....*XI. Lysis Inhibition*
39....*XII. Lysis from Without*

1. *Introduction.*—At first sight, the life cycle of a phage particle seems simple. It attaches itself to a bacterium and multiplies inside the bacterium. After a short time, something like 20 minutes, the bacterium bursts and several hundred phage particles are released. In the past, this life cycle has been viewed from the perspective of two distinct analogues:

(1) The enzyme precursor analogue. The phage particle is looked upon as a complex nucleo-protein molecule. The bacterium is assumed to contain an ample supply of precursor molecules, so similar to the infecting particle that the precursor can be transformed into phage by a relatively simple reaction, analogous to the conversion of trypsinogen into trypsin.

(2) The intracellular parasite analogue. The phage is looked upon as a micro-organism with exceedingly exacting growth requirements which can be satisfied only inside living cells. Specifically, one imagines that the phage has lost its entire assimilatory equipment. It therefore utilizes not only nutrients to be found inside the bacterium, but the whole organized enzyme equipment.

Phage research of the last decade has shown that both of these points of view must be far off the mark. The precursor view is incompatible with the variety of types that can multiply on one and the same bacterium and it is also incompatible with the fact that most of the substance of the new phage is derived from material assimilated after infection. The intracellular parasite view is contradicted by the genetic recombination data and by the various lines of evidence which point to a far-reaching disintegration of the infecting particle. The infecting particle loses its identity after entering its host. It merges with the bacterial organization and with other phage particles which

infected the same host. This new entity, the phage-bacterial complex, is then capable of bringing forth the production of several hundred new phage particles.

There is no good biological analogue for this phenomenon, and small wonder, since we are dealing with intracellular functional organization, which only genetics has tried to probe. In Luria's article, the specific arguments for this new point of view have been set forth in a coherent fashion. In this syllabus, we propose to explain in somewhat greater detail the procedures and facts of phage so as to permit an easier appreciation of the general argument, and as a guide to the literature.

I. Free Phage

2. We begin with a description of various features which characterize the phages in their extracellular existence, as *free phages*. Some of these are physical, physico-chemical, and chemical characteristics. Some others are biological characteristics, like host range and activation by cofactors, which show up only in the interaction of the phages with their hosts. They nevertheless serve to distinguish different states in which a phage particle outside the bacterium may be found.

3. *Identification of infective units with physical particles.*—There are five different methods for measuring the titer of an infective phage suspension. Three of these methods are biological, viz.:

(1) Titration by serial dilution to the limit of activity.

(2) Titration by plaque count.

It has been shown that the number of plaques is exactly proportional to the inverse of the dilution factor over a wide range of concentration (Ellis and Delbrück, 1939).

(3) Titration by the killing of bacteria.

Counting the number of surviving bacteria in an infected culture and applying Poisson's formula one obtains the average number of phage adsorbed per bacterium and thus the total number of phages, once the original number of bacteria is known (Luria and Dulbecco, 1949).

The fourth and fifth methods are physical.

(4) Electron microscope counting of particles.

Certain suspensions of polystyrene latex are made of very small spherical particles all of exactly the same diameter (Backus and Wil-

liams, 1948). This fact allows the determination of the absolute concentration of particles of latex by simple weighing. Luria and Williams (personal communication) made electron microscope preparations of a mixture of phage with latex suspensions of known concentration. They then counted the number of phage particles relative to the number of latex spheres, thus obtaining the relative titer of the phage suspension.

The results of the three biological methods usually agree with one another. They also agree with the physical method within a factor of two (T2 gave twice as many particles in the electron microscope as there were infective units measured by biological methods, T4 gave 20% less, and T6 30% more particles than infective units).

Since these four essentially different methods give the same result we may call the titer they give the absolute concentration.

Sometimes, however, for a strain of bacteria different from the normal host strain the plaque count may be low. We then speak of the strain as having a low efficiency of plating.

(5) Dark-field microscope visibility.

The scattering of light by phage particles renders them visible in the dark-field microscope. To see the smaller phages T1, T3, T7 a much stronger source of light is needed than for the larger ones, T2, T4, T5, T6. It is possible to estimate roughly the concentration of a suspension by looking at it in the microscope. The adsorption of a single phage on a bacterium is difficult to observe because of the violent Brownian motion of the particles, but one can follow the adsorption as a whole by watching the gradual disappearance of the phages when mixed with bacteria under the microscope. At the time of lysis the bacteria burst open liberating several hundred particles having the same scattering power as the infecting phages. (The liberation of particles upon lysis by T1 cannot be seen unless a very strong source of light is used.) These liberated particles disappear slowly from the field of view by diffusion and adsorption on other bacteria. There is thus no doubt that they are the infective units (Weigle, unpublished).

4. *Morphology.*

(a) Electron micrographs.

As seen in the electron microscope the phages have the following aspects.

T1: Round head, 45 to 50 mμ diameter, of uniform opacity.

Slender tail 150 mμ long, not more than 15 mμ thick. Tail appears straight or slightly curved (Wyckoff, 1949; Luria and Williams, personal communication).

T2: Large head 65 x 80 mμ, oval or in the form of a short rod with conical or rounded ends. Slender straight tail 120 mμ long and 20 mμ thick, terminating in a slightly broader part. There seems to be an internal structure in the head (Hook et al., 1946).

T4, T6 are indistinguishable from T2. All three show "ghosts" in older suspensions, and in preparations washed in distilled water (osmotic rupture), and in preparations treated with NaOH, or with sonic vibrations, or heavily irradiated with UV. These ghosts seem to be empty membranes (see below: Osmotic shock) (Anderson, 1949).

T5: Round head of 100 mμ diameter, distinct tail (Anderson, 1946).

T3: Round head of 45 mμ diameter, no tail (Anderson, 1946).

T7: Round (but not smooth) head of 51 mμ. The heads seem to contain a bilobular structure. Except for this, the particles seem essentially homogeneous without peripheral material. No tail (Kerby et al., 1949).

It has been claimed that T2 grown in synthetic medium is larger than T2 grown in broth (about 10%) (Sharp et al., 1946), that T3 has a diameter of 35 mμ, that both T3 and T7 show very slender tails in some preparations (Wyckoff, 1949), and that the diameter of T7 is 73 mμ when chromium shadowed (Sharp et al., 1949).

One must not forget that all the preparations seen in the electron microscope have been radically dried.

Indirect methods (sedimentation in centrifuge, diffusion, etc.) have given values for the dimensions of the phages compatible with those of the electron microscope.

(b) Osmotic Shock.

T2, T4, T6, which appear in the electron micrographs to have membranes, can be disintegrated by "osmotic shock." "Osmotic shock" is produced by incubation of the viruses in 4M NaCl, followed by a *rapid* dilution of the suspension in distilled water. The viruses are inactivated by this process, and electron micrographs of the suspensions show "ghosts," i.e., empty head membranes with tails still attached. T1, T3, T7 show no membrane in the electron microscope and are not

affected by osmotic shock (Anderson, 1949). Viruses treated by intense sonic vibrations show the same effect (Anderson et al., 1948).

5. *Chemical Composition of Phages.*—Biochemical analytical work has been done on T2 (Cohen, 1946b; Taylor, 1946), T4 (Polson et al., 1948; Cohen, 1946a), T6 (Putnam et al., 1950), and T7 (Kerby et al., 1949). The simplest and most reliable method for purification of phages is differential centrifugation of lysates at low and high speed. Uniformity of preparations is controlled by electron micrographs or in the analytical ultracentrifuge. In many cases it is difficult to be sure of dealing with homogeneous material especially where the samples taken are small. Generally, proteins and great amounts of nucleic acid are found in phages, the nucleic acid consisting entirely, or almost entirely, of DNA. The quantitative composition of a bacteriophage seems to vary with the medium in which the infected cells were kept. Phage particles of a given strain apparently are not merely uniform molecules of a highly complicated chemical substance. For this reason alone, a detailed comparison of quantitative differences in composition between different phage strains would not be very significant, let alone our lack of knowledge about the possible meaning of such differences. The amino acid content of T4 showed no striking deviations from that of the host *E. coli* (Polson and Wyckoff, 1948). A significant distinction, however, exists in the nucleic acid type, the ribopentose type being predominant in the bacterium.

6. *Stability.*—There are many factors which affect the stability of phages. Temperature, pressure, chemical action, irradiation, and ionic environment are just a few of these. Studies have been confined mostly to the conditions used in experimental work with phages.

The most detailed study is by Adams (1949).

The seven T phages are relatively stable in broth. At temperatures up to 50°C the rate of inactivation is hardly measurable. Above 50°C, the rate increases. The kinetics of inactivation is of the first order.

In *dilute sodium salt solutions* most of the phages are inactivated at a measurable rate at room temperatures. For example, at 20°C in 0.1N NaCl, about 78% of phage T5 is inactivated in 60 minutes, and at 30°C about 95% of this phage is inactivated during the same time in the same concentration of sodium ions. The rates of inactivation are not significantly different, whether the anion is chloride, citrate, or phosphate. As the concentration of sodium ions is increased above .1N, the inactivation rate of the phages is decreased rapidly. At a concentration of sodium ions of 1N, the rate has decreased by a factor of 10^7 and phage T5 is then as stable as it is in broth.

The inactivation of T_5 and of the other phages can be prevented by any one of a number of divalent cations at much lower concentration than the stabilizing concentration of sodium ion. Tests have shown that all metal divalent cations have a protective effect for phage except lead and mercury. The concentration which offers maximum protection is 10^{-3} M. Calcium shows a considerable protective effect at 10^{-4} M. The change of stability with changing concentration of divalent cation is very much less violent than in the case of sodium ion.

The meaning of these effects of cations on stability is not well understood.

Studies have been made on the stability of phages at high pressures (Foster et al., 1949). The results will not be detailed here.

The inactivation rate of T_2 has not been studied accurately, because the inactivation curves often show irregularities which are difficult to reproduce and therefore difficult to analyze. This behavior might be due to clumping of phages in groups of two or more.

The effect of hydrogen ion concentration on the rate of inactivation has been measured for T_5 (Adams, 1949) and T_7 (Kerby et al., 1949). Over the range from pH 5.5 to 7.5, the rate of inactivation seems to be independent of hydrogen ion concentration, but below 5 and above 9, the rate of inactivation is greatly accelerated.

The seven T phages are rapidly inactivated at gas-liquid interfaces (Adams, 1948). The smaller phages, T_1, T_3, and T_7, are inactivated at a faster rate than the other phages. This inactivation is a physical inactivation rather than a chemical one. The rate of inactivation is not affected by varying the gas (nitrogen, oxygen, and carbon dioxide) with which the phage suspension is shaken or bubbled. The kinetics of this inactivation is also of the first order. This inactivation may be prevented by the addition of small amounts of protein. Of the various proteins studied, gelatin gives the best protective effect.

7. *Serology.*

Bacteriophages as antigens.—Bacteriophages are generally good antigens. They exhibit a rigorous serological autonomy.

(a) Antisera prepared against the bacterial hosts are inactive on the bacteriophages.

(b) Antiphage sera previously exhausted by the bacterial hosts retain their full activity towards the bacteriophage with which they have been prepared.

The serological specificity of a given strain of a bacteriophage is retained by it on whatever bacterial strain it has propagated. This specificity is not related to the host range activity of the bacteriophages. Strains of phages active on the same bacterium may belong to very different serological groups. For instance, the seven phages of the T series, all active on *E. coli*, strain B, have been shown to belong to four serological groups (see Table I, p. 147). Phages of the same group cross-react with a serum prepared against any one of them. The titer of such a serum is generally 5 to 100 times lower when tested with an heterologous strain than with the homologous one.

The serological classification of phages has proved to be the only significant one (Burnet et al., 1937; Delbrück, 1946). Phages which belong to the same serological class are generally closely related as far as size, shape, and other morphological characters are concerned (cf. taxonomy). Mutations involving changes in plaque size, host range, etc., are well-known (cf. mutants), but no mutant of a given strain of bacteriophage has been found involving any detectable modifications in antigenic properties.

Phage anti-phage reactions.—In a mixture of virus and antiserum, the fraction of active virus decreases exponentially with time in many but not in all cases (Andrewes and Elford, 1933; Hershey, 1943). The rate of inactivation is proportional to the concentration of the serum.

The rate constant of inactivation is a characteristic of the activity of a given antiserum against a given phage. A good antiserum will inactivate 99% of a phage suspension in one minute at a dilution 1:100.

The combination of antibody with phage is irreversible in the sense that the complex is not dissociated upon dilution. The activity of neutralized phage, however, appears to be recoverable by treatment of the complex with papain (Kalmanson and Bronfenbrenner, 1943). Serum inactivated T3 can also be recovered by treatment with sonic vibrations (Doermann and Anderson, 1950, unpublished).

It has been shown that a bacteriophage particle is able to adsorb up to 5,000 molecules of antibody, but an adsorption of an average of 90 molecules is sufficient to suppress reproduction of the virus. Since the inactivation curve is approximately a one-hit curve one has to imagine that of the 90 antibody molecules adsorbed at the time of inactivation only one or two are adsorbed to really crucial spots on the phage. Just barely neutralized particles are still able to adsorb onto

the sensitive bacterium (Hershey, 1948). In this case, the presence of the antibody interferes with some step in the multiplication of the virus subsequent to its attachment to the bacterial cell. Bacteriophages partially coated with antibody which did not attach to crucial spots not only adsorb but also reproduce (Burnet et al., 1937). To explain these results it has been proposed (Burnet, 1937) that there are two kinds of receptors on the phages: one for the antibody and one for the bacterial surface.

When a phage is adsorbed on the sensitive host, subsequent treatment with antiserum no longer interferes with its multiplication (Delbrück, 1945a). The latent period and the burst size are unimpaired. This fact has proved to be very useful in permitting a precise estimate of the amount of free and adsorbed phage. If the unadsorbed phage of an adsorption mixture has been neutralized by addition of antiphage return, the ratio of the plaque count found after addition of the serum to that present initially is then the fraction of the infective centers due to adsorbed phage.

Experiments by Burnet have shown that formalin-killed bacteria heavily coated with phage are agglutinated by the homologous antiphage serum but are not agglutinated by heterologous serum. This indicates that the phage is still able to react with antiserum when adsorbed to a killed bacterium.

8. *Virus mutants.*—All mutations to be considered are spontaneous mutations, no reliable data on *induced* mutations are available. All mutations to be considered occur (presumably) *during multiplication* of the phages, not in stored stocks.

The important mutants are:

(1) Host range mutants, designated by the letter h (Luria, 1945; Hershey, 1946a).

(2) A class of plaque-type mutants, giving a larger plaque than the wild type, with a clear instead of a turbid halo. This type is designated by the letter r (Hershey, 1946a, b). There are many genetically distinct, but phenotypically similar mutants of the r type, and these are distinguished when necessary by numerals following the r (Hershey and Rotman, 1948).

Thus: T2 means the wild type of this strain.

T2h means its host range mutant, in most cases a mutant that can multiply on B/2 (cf. glossary).

T2r means a mutant giving larger plaques than the wild type, with a clear halo.

T2r7 means a seventh distinct mutant of this type.

T2r7r13 means a phage known to carry the r mutation in the two indicated loci. (Obtained by a suitable genetic cross.)

T2hr7 means a phage carrying both the h and the r7 mutation.

If it is desired to indicate specifically that a phage does *not* carry a mutation in a given locus, this is indicated by adding to the symbol of the strain the symbol of the mutation with a superscript $^+$, thus, T2h$^+$r13 is a mutant which carries the wild type allele at the h locus and the mutant allele at the r13 locus.

There are several wild type strains of T2 in use which are distinguished by capital letters. The principal ones are T2H and T2L.

Another class of mutations, "biochemical mutations," has been noted (Anderson, 1948; Delbrück, 1948). The mutational patterns have not yet been worked out. They have also not yet been used systematically in recombination studies.

The idea of distinct "genes," or "loci," on "genetic sites" in phages originated with Hershey's studies of the *mutational pattern* of T2 (Hershey, 1946a,b). Hershey utilized several mutations besides those listed above and demonstrated that each of these mutational steps occurs independently of the state of the phage with respect to other mutations.

9. *Taxonomy.*—The principal index of relatedness between phages is their serological cross reaction. By this criterion the seven phages of the T series fall into four groups, as follows:

T1; T3, T7; T5; and T2, T4, T6 (the even numbered phages). Phages in different groups do not cross react at all. Within a group they do cross react (see 7). Within each group, the phages are morphologically alike, phages belonging to different groups are morphologically distinct (see Table I, p. 147).

Contrary to earlier beliefs, host range is *not* a useful taxonomic feature. Thus, T3 and T4 have a very similar host range, although they are unrelated by every other index. On the other hand, T2 and T2h, which differ in host range, are obviously very closely related.

Plaque type is of taxonomic value if applied cautiously. Generally speaking, small phages give large plaques, but plaque sizes are affected also by mutations.

The even numbered phages represent the group of widest distribution. Many of the coli phages studied by earlier workers belong to this group.

10. *Cofactor Activation.*—(T. F. Anderson, 1945, 1948b; Hershey and Delbrück, unpublished; Wollman and Stent, unpublished.)

It has been found that T4 and T6 exist in two states, "active" and "inactive," depending on the presence of certain amino acids, called cofactors. If suspended in cofactor-free synthetic medium, such phages will be in the inactive state and will not be adsorbed to their host bacteria. When cofactor of sufficient concentration is added, the phages become active and are then able to adsorb on, and later lyse, the host cells.

L-tryptophan is the most active cofactor found thus far, and its action has been studied in the greatest detail. Other aromatic amino acids, such as phenylalanine, show less activity. Only the L-isomers appear to be effective. Indole is an inhibitor to the activation by L-tryptophan (Delbrück, 1948 and unpublished), and it is assumed that competition occurs between cofactor and inhibitor at specific sites on the phage.

It has been estimated that the number of cofactor molecules associated with each phage particle is of the order of two hundred.

The process of activation is reversible, and the reaction velocities with which gain or loss of activity occurs upon addition or removal of cofactor can be studied. The following results were found:

(1) Activation and deactivation appear to follow first order kinetics and to be "one-hit" processes.

(2) The rate of activation varies approximately with the fifth power of the cofactor concentration at low concentrations of cofactor and is independent of the cofactor concentration at high cofactor concentration.

(3) The rate of deactivation is greatly retarded by concentrations of cofactor which cause only very little activation if mixed with inactive phage.

A model of the activation mechanism has been formulated which ties together these findings. The phage is thought to contain key places which can be in either an active or an inactive state. Each phage has either just one or a large number of such key places. The key places become active by a reaction involving the simultaneous presence of

five cofactor molecules. At low cofactor concentrations, when only a small fraction of the key places have the necessary five cofactor molecules, the number of key places gaining activity per unit time will vary with the fifth power of the cofactor concentration. At high cofactor concentrations, when all key places have at least five cofactor molecules within them, the rate of activation will be independent of the cofactor concentration. Deactivation is thought to occur when one of the five cofactor molecules from an active key place breaks loose. It is, however, possible for another, exogenous cofactor molecule to take the place of the lost one if it can react in time with the remaining four molecules of a recently deactivated key place. Thus, the efficiency of low cofactor concentrations in retarding deactivation depends on the first power and not, as is true in the case of activation, on the fifth power of the cofactor concentration.

The efficiency of a given low cofactor concentration in activating the phage is drastically reduced by lowering the temperature. This is readily understood in terms of the above model. If the probability of finding a cofactor molecule within a key place at a given cofactor concentration is slightly less at a lower temperature, say, because of a weak bond being formed less readily, then this reduction makes itself felt to the fifth power in the observed activation rate.

11. *Radiation Inactivation.* A phage particle is defined as *inactive* when it is not able to give rise to phage *growth;* ordinarily this ability is tested by plaque formation. The inactivating influence of radiation is studied by determining *survival curves*, i.e. curves obtained by plotting the logarithm of the fraction of particles active after a dose D of radiation. Frequently this curve is a straight line indicating that the inactivation is the result of a single event ("hit"). In these cases, a dose which gives a survival of e^{-1} (0.367) is called the "inactivation dose" with the negative of the natural logarithm of the surviving fraction giving the average number of *hits* per particle.

(a) *Inactivation by X-rays.* Two types of inactivation. By *direct* and by *indirect effect* (Luria and Exner, 1941; Watson, 1950).

(1) *Inactivation by Direct Effect.* This refers to inactivation due to absorption of energy within the phage particle. It is obtained when phage is suspended during irradiation in a protective medium (broth, gelatin solution, tryptophane solution, etc.). The survival curve is perfectly straight indicating a one hit inactivating mechanism. The rate of inactivation is dependent on dose only. It is independent of intensity, fractionation of dose, the presence of oxygen, and the temperature during irradiation. The quantum

yield per ionization is considerably below unity; a recent determination for T2 gave a yield of 1/20 (Watson, 1950).

(2) *Inactivation by Indirect Effect.* This is due to toxic agents produced by the radiation in the surrounding medium and can be obtained when phage is suspended during irradiation in a medium free of protective substances. In this case inactivation by indirect agents greatly exceeds direct inactivation. Indirect inactivation is caused by at least two different agents or groups of agents: one, short lived, is detectable only by its action during actual exposure of the phage suspension to radiation; the other, relatively stable, is detected by the persistence of its effect after irradiation (Watson, 1950).

The survival curve due to the short lived indirect agent is not straight but shows a downward concavity at low doses becoming

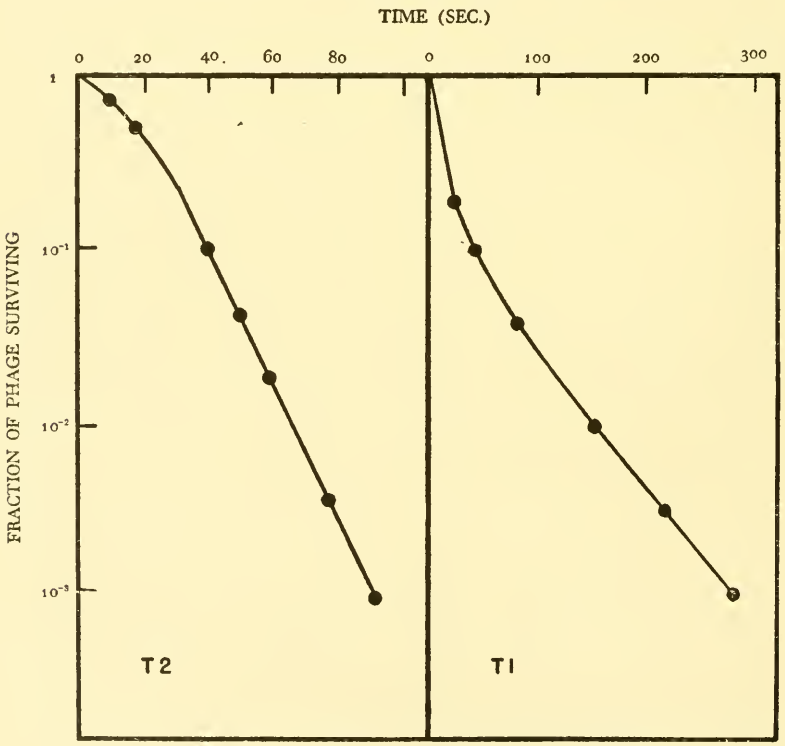


Fig. 1. Inactivation of phages T2 and T1 by UV irradiation. The log of the fraction of surviving phage particles as a function of the time of irradiation at a distance of 82 cm from a G.E. germicidal lamp.

straight at higher doses. The curvature is due to a stepwise killing of the phage (Watson, 1950). The rate of inactivation is independent of oxygen present during irradiation (Doermann, personal communication).

The presence of long lived indirect agents is shown by the inactivation of phage introduced into previously irradiated solutions. The rate of inactivation is strongly influenced by such variables as temperature and concentration of Cl^- ion (Watson, 1950).

(b) *Inactivation by Ultraviolet radiation* (UV). Inactivation of phages suspended in water or in a buffer solution is produced by light of wave lengths of 3130A or shorter. An exceedingly slight inactivation by light of wave length 3600A or longer has been reported by Wahl and Latarjet (1947). The action spectrum for inactivation is similar to the absorption spectrum, and the quantum yield at 2537A is of the order of 10^{-4} (Zelle and Hollaender, personal communication).

The inactivation curves of the seven phages of the T group approach straight lines for high doses of UV (Luria and Latarjet, 1947); at low doses the inactivation curves of phages T2, T4, T5, T6 show a downward concavity, and those of phages T1, T3, T7 show an upward concavity (see Fig. 1). The origin of these curvatures is unknown (Dulbecco, 1950).

Inactivation by UV in dried condition has been obtained for phage T1 (Fluke and Pollard, 1949).

UV inactivation is dependent on dose only; it is independent of intensity, fractionation of the dose, temperature during irradiation.

(c) *Inactivation by decay of P^{32} incorporated in phage structure* (Hershey, personal communication). A particle of phage T2 is inactivated on the average by one out of every 10 P^{32} transmutations occurring in its structure. Only a small fraction of this inactivation can be attributed to the beta particles emitted during transmutation.

II. Glossary

12. To describe the standard methods of handling phages a certain number of terms have been introduced which we now define.

When working with one bacterial strain and one phage strain conditions can be chosen so that most of the infected bacteria of a given culture are infected with only *one* phage particle or with more than one. The first case is called *single infection*, the second *multiple infection*, and the ratio of adsorbed phage particles to bacteria in the culture is named the *multiplicity of infection*.

There are no means of introducing directly an exact and desired number of phages into individual bacteria. Infection is brought about by mixing a suspension of a known number of bacteria with a suspension of a known number of phages. The proportion of these two numbers determines roughly the multiplicity of infection. Since adsorption of phages is never 100%, the actual multiplicity has to be determined for each experiment, for instance by comparison of the plaque count after adsorption with the total virus input. Each bacterium does not receive exactly the same number of infecting phage particles. Because of statistical fluctuations some will have more, some less. This kind of fluctuation is governed by the *Poisson Law* $p(r)=n^r e^{-n}/r!$, which gives the probability $p(r)$ of r objects being present in a given sample, when the average number of objects per sample is n. If, for example, $n=4$, the proportion of bacteria which have been infected by exactly $r=4$ phage particles is $p(4)=4^4e^{-4}/4!=0.2$. Only 20% of all bacteria have been infected with exactly 4 phage particles. In a similar way the percentages for all values of r can be calculated. Besides infecting bacteria singly or multiply with only one type of phage one can also try to infect them with two or more different phage strains. This is called *mixed infection.* The true conditions of such an experiment again have to be determined and calculated in the same manner indicated above.

For the analysis of mixed infection experiments, *indicator strains* of bacteria are used, which are resistant to certain phages. They are derived as mutants from the *E. coli* strain B which is commonly used in all these experiments and which is sensitive to all the seven T phages. The resistance pattern of each mutant is indicated in the following manner: B/2 means a strain resistant to T2; B/3, 4, 7 means a strain resistant to T3, T4, and T7, etc.

A mixture of T2 and T4 will show only the plaques of T4 when plated on B/2 and only those of T2 on B/4. *If B/2 and B/4 are mixed* and used for plating a mixture of T2 and T4, both phage strains will form plaques on the same plate, but the plaques will generally be turbid, since in each of them only one of the bacterial strains present is eliminated by lysis. But where the areas of a T2 and a T4 plaque overlap, a clear zone will be formed, since there both bacterial strains are lysed. Completely clear plaques are formed on *mixed indicators* by bacteria which have been mixedly infected and yield a mixed progeny of phages, since in such a plaque both indicators are lysed.

For experiments involving a phage and its host range mutants it is useful to employ a mixture of B and an indicator strain. Here the

wild type forms a *turbid plaque* and the *host range mutant a clear plaque.*

To determine the time between infection and lysis and the average phage yield of singly or multiply infected bacteria, the *one step growth technique* is used. After mixing bacteria and phages in the desired proportion, and allowing a few minutes for adsorption, unadsorbed or free phage (and only those!) may be inactivated by anti-phage serum. The mixture is thereupon highly diluted so that a sample used for plating contains only a few hundred infected bacteria. Each infected bacterium gives a single plaque if it did not burst before plating. In case the free phage were not inactivated previously by antiserum, there would be two indistinguishable kinds of plaques from two kinds of "infective centers," i.e., the infected and not yet lysed bacteria and the free phages. Plating samples of this diluted mixture at short time intervals give constant plaque counts for a certain length of time, then suddenly the count rises from plate to plate until it reaches a new, much higher level. The sudden rise in count is brought about by the first bursts of infected bacteria, which distribute the phages produced by them evenly throughout the dilution tube, increasing thereby the number of infective centers, since each newly released phage particle can now form a plaque of its own. The count levels off after the last infected bacterium has burst. The time interval between infection and the beginning of lysis is called the *latent period*, the time between beginning and end of lysis the *rise period*, and the ratio of the count at the end to that at the beginning the *step size*. From the latter it is easy to calculate the *average burst size* of an infected bacterium. The whole experiment can be plotted on graph paper (count vs. time) in order to obtain a *one step growth curve.*

Individual burst sizes are determined by another type of experiment where the infected bacterial suspension is diluted so far that a single drop of the suspension contains on the average much less than one bacterium. Distributing drops into a great number of tubes and plating each tube after the latent period is over, gives no plaques at all for most of them, the rest containing mostly the yields of single bursts. Again the Poisson Law serves for detailed analysis of this *single burst experiment.*

In many experiments it is necessary to count free phage and infected bacteria separately. This can be done either by phage antiserum, which, as indicated above, inactivates only unadsorbed phages, or by short centrifugation, which throws down bacteria and leaves free phage in the supernatant.

III. Adsorption

13. The term "adsorption" refers to that part of the life cycle in which phage and bacterium form an irreversible union. Experiments with X-rayed viruses (see 14) demonstrate that this step is empirically recognizable, and that it involves certain side effects (see 38, 39).

Whether or not such a union can occur between a particular phage and bacterium depends on highly specific factors. A host strain normally sensitive to one particular type of phage may mutate to resistance (i. e. to viability in the presence of the virus) while retaining its sensitivity to other phage types. Such a bacterial strain no longer forms an irreversible bond with the phage to which it has become resistant. Sensitive bacteria, on the other hand, can unite with the phage even under conditions where no reproduction of the phage is possible. Adsorption proceeds, for example, when phage and bacteria are suspended in buffer containing no metabolic substrates. If either virus or cell has been mistreated in some way, such as X-ray or ultraviolet irradiation of the phage, or heat- or alcohol-killing of the bacteria, adsorption still occurs. It is, in fact, possible to make extracts from bacteria which can unite irreversibly and specificially *in vitro* with active phages (see 17).

The adsorption process involves, therefore, some specific structures on the surface of host and virus, perhaps analogous to those responsible for the steric fitting encountered in antibody antigen reaction. It does not involve metabolic processes on the part of the bacterium.

14. *Adsorption without killing of the bacterium.*—It has been discovered by Watson (1950) that a large fraction of the phage particles inactivated with X-rays is adsorbed by the bacteria without killing them. Proof that they are actually adsorbed is obtained by showing that these particles can saturate the *adsorptive capacity* of the bacteria. Bacteria killed by heat (one hour at 60°C) are able to adsorb particles, up to a maximum of about 500 for each bacterium; Watson could show that heat-killed bacteria can have their adsorptive capacity saturated by exposure to phage inactivated by X-rays. He demonstrated this saturation by the subsequent reduction of their adsorptive capacity for active phage.

15. *Mechanism of the adsorption process.*—A study of the velocity with which active phages are adsorbed to sensitive bacterial cells shows that the fraction of the phages remaining free decreases exponentially with time. In a certain range of bacterial concentrations, moreover, the adsorption rate is proportional to the cell concentration. We con-

clude, therefore, that adsorption is a pseudo first order reaction, i.e., that there occurs a collision of two bodies, one of which (the bacterial receptors) is in large excess over the other (the phage).

In case neither bacteria nor phage have motility, one may calculate the approximate frequency of collision of the two bodies due to Brownian movement in suspensions of known concentration (Schlesinger, 1932). This calculated frequency is of the same order of magnitude as the rate of adsorption observed under the most favorable conditions. Thus, almost every collision between phage and host must lead to specific fixation.

It is possible to show, however, that under other conditions active phage may collide with sensitive bacteria without being adsorbed. Experiments with starved bacteria (Delbrück, 1940a) and at low temperatures (Wollman and Stent, unpublished) show that the reduction in the adsorption rate observed is much more severe than that predicted from the decrease of the collision frequency. Under *unfavorable* conditions therefore, the fraction of the collisions leading to adsorption can be greatly reduced.

It is found that the adsorption rate of phages no longer increases with the bacterial concentration after the cell density has exceeded a certain limit. (Anderson, 1949; and Wollman and Stent, unpublished). Since the collision rate should continue to increase under these conditions, one is led to postulate a second, rate limiting, step of the adsorption reaction.

The finding that no adsorption at all occurs in mixtures which are agitated violently (Anderson, 1949) can be interpreted to mean that stirring prevents phage and host from staying together long enough to undergo steric fitting.

16. *The bacterial change from sensitivity to resistance.*—Bacteria can change by mutation from a form in which they are sensitive to a given phage to a form in which they are resistant to it. *By resistance we mean here that the viability of the bacterium is not affected by the phage.* The word is sometimes used also in a less stringent sense. For instance, in the sense that the bacterium has become unsuitable for multiplication of the phage although it is still attacked and even killed by it.

In strain B and the phages of the T series, *resistance in the strict sense is always associated with failure of the resistant bacteria to adsorb the phage to which they are resistant.* These resistant mutants are obtained by plating out 100 or 1,000 million bacteria of the sensitive

strain in the presence of an excess of the phage. The sensitive bacteria are thereby destroyed, the resistant ones survive and form colonies.

Since the resistant mutants so obtained always fail to adsorb the phage to which they have become resistant, it is reasonable to assume (but difficult to prove) that the resistance is due *solely* to the failure of the adsorption mechanism. One may imagine that the mutation has led to the formation of altered surface elements unable to adsorb the phage. This point of view will be elaborated in more detail in the next section.

In one instance, we have suggestive evidence that it is really only the adsorption mechanism whose failure makes the bacterium resistant, and not a failure of the ability of the bacterium to synthesize the phage in question. This evidence is derived from the following case: When bacterium B is mixedly infected with T2 and T4, there results particles like the parental types, then some genetic hybrids as will be described later (32), and in addition, particles of a peculiar type, which will be designated as T2(4). Such particles have the following properties (Szilard and Novick, personal communication; Delbrück, unpublished):

1) They are adsorbed by B/2 as if they were T4 particles.

2) They multiply within B/2, but their offspring consists entirely of T2 particles.

The T2(4) particles are interpreted as having the genotype of T2, but phenotypically they have some of the T4 properties, and it is these T4 properties which enable the particles to adsorb to and enter B/2 bacteria.

This case is important in that it shows that B/2 which cannot adsorb T2 can nevertheless reproduce T2 when it is infected with a suitably equipped T2 particle. The adsorptive abilities of the bacterium, therefore, do not necessarily constitute a complete display of the synthetic or reproductive abilities of a bacterium.

17. *Receptor Spots.*—The close resemblance between neutralization of an antigen by an antibody and the adsorption of a phage on to a sensitive bacterium led to early attempts to isolate substances from bacteria, which would represent the "receptor spots" of the bacterial surface responsible for the specific attachment of the infecting phage particle to the sensitive cell. *In vitro* the reaction between receptor substance and phage should lead to an inactivation of the phage, which could serve as a test in the course of purification procedures. Indeed it is possible to obtain extracts from bacteria, which inactivate specifically phages infective for the extracted bacterial strain (Beumer, 1947).

Attempts for further purification of the active extracts have in most cases not been made, probably because loss of activity is frequently encountered.

17a. *Supplementary information on receptor spots*, by *W. Weidel*

Preparation of receptor material

Bacteria from a 15 l. broth culture grown to near saturation are spun down in a Sharples Supercentrifuge and resuspended in isotonic Michaelis buffer (sodium veronal sodium acetate-HCl) of pH 8.2 (250 cc). A few cc of toluene are added and the suspension is kept at 37°C with slight aeration for 24 hours. After a few hours the pH is controlled and readjusted to 8.2 if necessary. Toluene lost by aeration has to be replaced.

After centrifugation in an angle-centrifuge the clear, yellow supernatant is discarded and the sediment washed thoroughly twice with 100% alcohol on the centrifuge. Two washings with 0.85% NaCl follow in order to remove the alcohol. The sediment is resuspended in a solution of trypsin (4 g) in 250 cc of Michaelis buffer of pH 8.2.

> Commercial trypsin is suspended in the amount of water required for dilution of the concentrated buffer mixture, the suspension is slightly acidified with N-HCl (Congo paper faintly purple) and centrifuged. The decanted supernate is then adjusted to pH 8.2 with N-NaOH, combined with the concentrated buffer mixture and the whole liquid used for resuspension of the bacterial sediment mentioned above.

Trypsin digestion is carried out at 37°C under toluene for 48 hours. After high speed centrifugation (12,000 rpm in Sorvall centrifuge) the clear supernatant is discarded and the sediment, which usually consists of two layers of different appearance, is resuspended in 125 cc of Michaelis buffer of pH 5.2; 2-3 mg of crystallized lysozyme and a few cc of toleune are added and the mixture is then incubated at 37° C for 24 hours. Following this treatment, the pH of the mixture is switched to 8.2 with N-NaOH and a solution of 2 g trypsin in 50 cc of water, purified as described above, is added. After incubation for 24-48 hours under toluene at 37° C some heavy material is removed by low speed centrifugation (3,000 rpm) for 15 minutes. The decanted, greyish, strongly light scattering supernatant is thereupon centrifuged at high speed, yielding an almost clear supernatant and a grey paste, which in strong light is completely transparent, showing a yellow-brown color. This material is washed several times on the high speed centrifuge with

distilled water. Small amounts of non-transparent material on the bottom of the centrifuge tube are removed mechanically. 50 g of moist bacteria yield about 25 g of sediment, the dry weight of which amounts to only about 10% of the dry weight of the bacteria.

*Morphology**

Electron micrographs show that the sediment prepared by the method described here consists entirely of bacterial cell walls, which are crumpled up and folded to give completely flat, almost circular particles of rather uniform size. The uniformity of the material is confirmed by studies in the ultracentrifuge and by electrophoresis, where sharp boundaries and no indication of major impurities were seen.

If the preparation is made from bacteria, which have begun to come into the lag phase, one obtains not only this material but in addition bacterial cell walls which did not fold up to give disk shaped particles but retained the oblong shape of the coli cell. This is also evident macroscopically by the strong streaming birefringence shown by this fraction which can be separated from the usual folded membranes by differential centrifugation. The stretched membranes require a much longer time to be spun down. Morphological details appearing on electromicrographs shall not be described here. It seems to be clear that the particles — stretched or folded — are nothing but empty collapsed bags with extremely thin walls.

Anti-phage activity

Both folded and stretched membranes, suspended in concentrations corresponding to bacterial cultures of 10^7 - 10^{10} bacteria per cc adsorb phages T_2, T_4, and T6 approximately as fast as normal living bacteria. The rates depend on the concentration of the membranes as they do on the bacterial concentration. The cofactor requiring strains of T_4 and T6 are adsorbed by the membranes in broth, or buffer plus tryptophan, but not in buffer alone. The adsorption capacity of the membranes for T6 seems to be smaller than that for T_4 and T_2 and it also seems to be dependent upon the age of the culture from which they were prepared. Very slight adsorption occurs with T_3 and T_7, none with T_1 and T_5.

Electron micrographs of mixtures of T_2 and membranes, which were kept overnight in the ice box, seem to indicate that the membranes

*The electron micrographs were taken in collaboration with Dr. R. F. Baker of the University of Southern California.

are dissolved by the phages. The membranes disintegrate into granular material.

A similar material, isolated by the same procedure from a B-mutant sensitive to T2 only, inactivates only T2 phages.

Chemistry

The bacterial membranes are built from an astonishingly resistant material. For example, organic solvents, low or high pH, saturated urea solutions and trichloroacetic acid, drying in the desiccator, have no damaging effect upon their macroscopic appearance or upon their antiphage activity. Hydrolysis with concentrated HCl at room temperature for several days yields a mixture of amino acids, a completely insoluble substance, as yet undefined, and a lipid fraction consisting of a neutral oil and a mixture of fatty acids, one of which is crystallized. The sodium salts of these acids gelatinize in water solution at high dilution. No reducing sugars could be detected in the hydrolysate, and no humin was formed during hydrolysis. Elementary analysis indicated the presence of about .5% phosphorous. Analysis from different preparations gave only minor fluctuations of the values for C, H, N, and P. From these data, which still have to be completed, one might draw the conclusion that the cell wall of coli B or a certain well defined layer of it consists of a phospholipoproteid of unusual properties. It has often been claimed that polysaccharide compounds of bacterial cell walls play a main role in specific adsorption of bacteriophages to their host. The results of this work do not support this assumption for *E. coli* B.

A somatic antigen of Sh. sonnei was isolated and showed specific activity against T3, T4, and T7 (Miller and Goebel, 1949), but the pure substance was found to be almost inactive after a few months. Chemically it is a lipocarbohydrate-protein complex. It seems that bacterial antigenic polysaccharides serve as the specific sites in many cases (Pirie, 1940).

IV. Invasion

18. In the preceding section we have seen that the specific combination of phage with its receptor spot does not harm the bacterium. The next phase that we can distinguish kills the bacterium. This stage is defined, as is the previous one, by certain freak situations in which progress beyond it is made impossible. We call this the stage of *invasion.*

19. *Characteristics of invasion.*—At this stage new infective particles have not yet been produced, nor will there be any produced if progress is arrested. On the other hand, the infecting particle is lost

and cannot be recovered by any known means. The bacterium is killed in the sense that it fails to proliferate and cannot be made to do so by any known means. The respiratory rate is not impaired, but it also does not increase, as it would in the uninfected bacterium (Cohen and Anderson, 1947; Monod and Wollman, 1947). The bacterium also has lost its ability to form adaptive enzymes (Monod and Wollman, 1947). A further characteristic feature of this stage is that mutual exclusion has become established (see 31). One has to imagine that at this stage the attacking virus and the bacterium have formed a functional unit designed for the production of phage but not necessarily able to do so. The cases in which progress gets arrested at this stage are the following:

20. *Phage T5 in the absence of calcium* (Adams, 1949).—In the absence of calcium, this phage is adsorbed and kills the bacterium, but no new phage is produced. If calcium is added later, lysis and phage liberation take place 40 minutes after the addition of calcium. Forty minutes is the normal latent period of this phage. The absence of calcium seems to block the development at a very early stage.

21. *Ultraviolet (UV) treated phage* is also arrested at the invasion stage, i.e., such phage is adsorbed and kills the bacterium (Luria and Delbrück, 1942) and excludes others (see 31). UV phage is of extreme interest because of the various reactivation phenomena associated with it, namely, *photoreactivation* described in 33, and *multiplicity reactivation* described in 34.

When phage is exposed to the direct effect of X-rays approximately 1 out of 3 "hits" destroys the ability of the phage to kill the bacteria. X-ray inactivated phage thus consists of two fractions: a "killing" fraction which kills the bacteria following adsorption, and a "non-killing" fraction which adsorbs without killing (Watson, 1950).

V. Multiplication

22. *Burst size distribution—correlation with size of bacteria.*—The yield of virus from an individual bacterium can be determined. It is called the burst size of the bacterium (see 12). One finds in all cases that the burst sizes vary enormously, from a few up to around 1,000, with a very broad maximum (Delbrück, 1945b). A part of this variability of the burst size may be due to variations in the sizes of the bacteria. In fact, from observations in the dark field microscope one does get the impression that larger bacteria liberate on the average more particles than do small bacteria. However, the variations in burst

size are certainly very much greater than the variations in bacterial volume.

23. *Latent period.*—The latent period between infection and lysis also varies from bacterium to bacterium, but to a much lesser degree than the burst size. The point that can be determined with greatest convenience and accuracy is the *minimum* latent period, and this is generally meant when we speak of the latent period. For the seven phages of the T series the latent periods are given in Table I (p. 147). These are the values obtained at 37°C, in nutrient broth, and with bacteria in their phase of most rapid division.

A very striking feature about the minimum latent period is that it is the same in the case of *multiple* infection as in *single* infection (Delbrück and Luria, 1942). This was first established in 1942 and has since been confirmed in every well-studied case. A bacterium infected with 10 phage particles does not seem to have a head start over one infected with only one phage particle. Doermann has broken up bacteria prior to their natural term of lysis and has found that multiply infected bacteria are very slightly ahead of singly infected bacteria opened at the same time (see 27).

24. *Influence of growth medium.*—If the growth medium is changed the latent period changes very little, even though the bacteria themselves may grow much more slowly in the new medium. Thus, the latent period of T2 in nutrient broth is 21 minutes. In this medium, the bacteria divide every 19 minutes. In a synthetic glucose medium, where the bacteria divide about every 40 minutes, the latent period of T2 is still 21 minutes. It is generally found that the burst sizes are smaller in simple media than in broth. One has to imagine that the slow growth of the bacteria is due to the higher complexity of synthetic operations required of a bacterium living in a simple medium. This complexity limits also the rate of synthesis of material that is to go into virus, thus decreasing the burst size, but the latent period is determined by internal factors, independent of assimilatory procedures.

25. *Dependence of the latent period on the temperature.*—A change of temperature, in contrast to a change of medium, changes the pace of *all* bacterial functions, not only the pace of assimilation. It is therefore not surprising to find that a change of temperature affects also the latent periods, roughly in proportion to the influence that temperature has on the division time of the uninfected bacteria (Ellis and Delbrück, 1939).

26. *Dependence of growth upon specific supplies.*—Two facts have been well established.

1) Phage multiplication depends upon the presence of oxidizable compounds in the suspending medium of infected cells. Phage multiplication does not occur in buffer solutions and does not occur if the respiration of the infected cells is blocked in one way or another.

2) Material from the medium is used not only for supply of energy but finds its way into the newly produced phage particles as well. This was shown by using the tracer technique with isotopic P, N, and C (Cohen, 1948; Putnam and Kozloff, 1950; Hershey, unpublished).

Detailed data are available now for radioactive phosphorous. It was found that 70 to 80% of the phosphorous content of T6 stems from the inorganic phosphate of the medium. Only the remaining 20 to 30% are derived from host material present at the time of infection. It should be mentioned here that apparently little of the phosphorus of the *infecting* phage particle or particles, which start the multiplication process, appears in their progeny. Most of it is found in the medium after lysis in low molecular form or in the bacterial debris (up to 78%). This observation supports once more the assumption that the infecting phage particles disintegrate and are not recovered as such.

Studies of the metabolic procedures involved in phage reproduction, their mutual interaction and timing, have been done without too much clarification. Various substances are capable of stopping multiplication reversibly (proflavin, 5-methyl-tryptophan) or irreversibly (depending strongly on time of removal of poison); other substances were found to be indispensable for virus growth (Cohen, 1949). It is not yet clear on which level such interactions occur. At least two alternatives are always possible: either some sub-unit of the virus itself is affected directly or the growth supporting metabolic machinery of the bacterium is altered, consequently affecting virus multiplication, but in a more indirect manner.

Cohen studied gross chemical changes occurring in T2-infected bacteria during the latent period. He found that protein synthesis continues immediately after infection, DNA-synthesis is delayed by seven to ten minutes but then goes on at a constant rate, RNA-synthesis is stopped. In uninfected cells RNA-synthesis exceeds that of DNA-synthesis about three times. Most of the material synthesized by infected cells appears to be phage material.

27. *Premature lysis.*—Multiplication of phage particles proceeds behind the cloak of the cell wall, and the result is not revealed until the

cell lyses, which does not occur spontaneously until many new particles have been formed.

Doermann (1948) and Doermann and Anderson (1950) have succeeded in disrupting the cell prematurely by the following methods:

1) Lysis from without (Delbrück, 1940): Bacteria infected with T4 are metabolically inhibited with 5-methyl-tryptophane (Cohen and Fowler, 1947) or cyanide and caused to lyse by means of a high concentration of T6. Plating is then done on B/6, so that only T4 is measured.

2) Lysis by addition of cyanide alone, which is effective in the later part of the latent period.

3) Disruption of infected cells by sonic vibration. T3 is used here, since it is relatively resistant to sonic inactivation (Anderson et al., 1948).

For the first half of the normal latent period, no viable phage is obtained; the original infecting particle is not recovered. Complete new particles begin to appear at roughly half time and the number appearing thereafter is a linear function of time until the maximum yield is reached (see figure 2).

According to the experiments of Foster (1948), proflavine inhibits a late phase in the synthesis of phage without preventing lysis, so that results similar to Doermann's are obtained by adding proflavine at a given time and allowing the cells to lyse spontaneously.

28. *Fate of the infecting particle.*—The infecting particle is lost; it is not present in the yield of the infected bacterium, and apparently the majority of its chemical constituents are not utilized for building new phage. Three lines of evidence lead to this conclusion:

A. In mixed infection with the phages T1 and T2, phage T1 is "excluded" (see 31); the mixed-infected bacterium yields only phage T2, and the T1 infecting particle does not appear in the yield (Delbrück and Luria, 1942).

B. The experiments on premature lysis (see 27) show that during the first one-half of the latent period no particles can be recovered (Doermann, 1948).

C. If a bacterium is infected with phage labelled with P^{32} a fraction only of the hot phosphorus present in the infecting particles can be recovered in the yield (Putnam and Kozloff, 1950). (This loss of P of

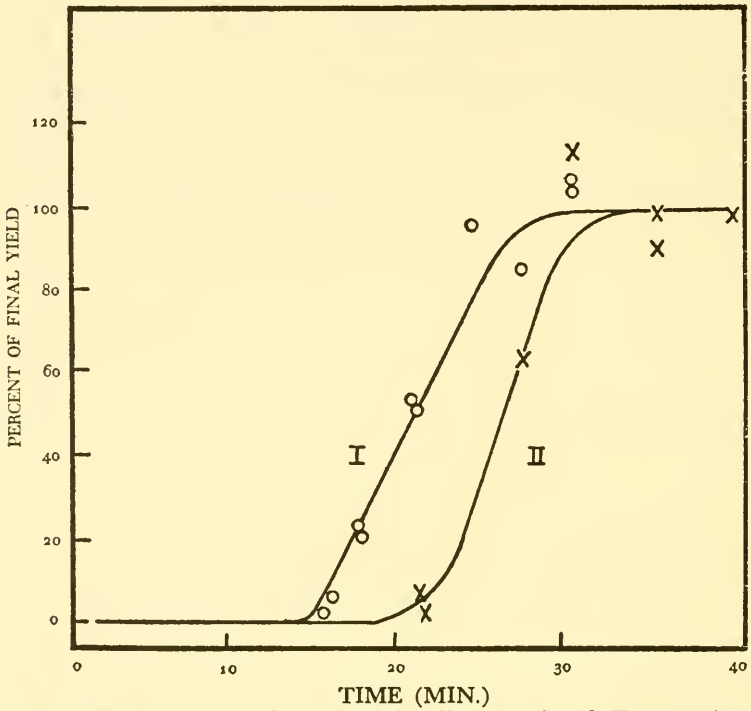


Fig. 2. Premature lysis of B/r/1 cells infected with T3, at various stages of the latent period. The percent of the final yield of phage as function of the time of incubation at 30C after infection.
Curve I: Infected cells disrupted after incubation by sonic vibration.
Curve II: Control one-step growth curve.

the infecting particles may however be due to secondary causes.) If, on the contrary, a bacterium is infected with high multiplicity of unlabelled phage and grown in medium containing P^{32}, the phage appearing in the yield shows the specific activity of the medium without dilution from the cold phosphorus of the infecting particles (Hershey, personal communication).

VI. Mixed Infection

29. *Mixed infection with particles differing by one mutational step.*—Experiments of this type were performed with the mixture T2+T2r. The yield of a bacterium infected with the two types contains particles belonging to both types. On the average the proportions in the yield are equal to the proportions among the infecting particles (Hershey, 1946).

If, among the infecting particles, the proportion of one type is considerably reduced, some of the bursting bacteria, although adsorbing both types, yield phage belonging to the majority type only. Quantitative analysis of this phenomenon shows that a limited number of phage particles can take part in growth in one bacterium, this number being of the order of 8 to 10 for phage T2 (*limited participation*, Dulbecco, 1949 a).

30. *Mixed infections with mutants carrying two genetic markers. Recombination. Linkage.*—We now turn to those mixed infections which require for their interpretation the idea of genetic recombination. We will begin with the case investigated most recently because it gives the clearest and the most detailed picture of the situation. This is the case of a mixed infection of a bacterium with a host range mutant (h) and a plaque mutant (r) of T2 (Hershey and Rotman, 1949).

From such a mixed infection there results four types of particles, two like the parental types and two recombinant types, designated as rh and ++ (wild type). These four types can be distinguished when platings are made on a mixture of B and B/2. (See photograph.)

If yields of individual bacteria are examined, it is found that almost every burst contains particles of each of the four types, which can be separately enumerated. Hershey and Rotman have done these experiments on a large scale, with the following principal results:

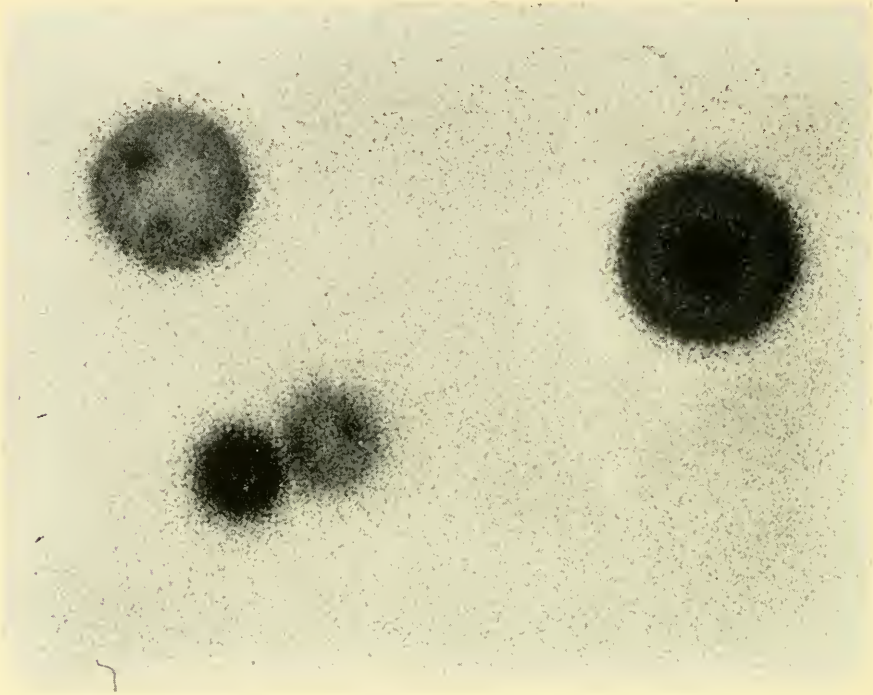
1) The average yields of the two parental types are alike.

2) The average yields of the two recombinant types are alike.

3) In individual bacteria the yields of the four types vary widely, just as the total burst sizes do.

4) The proportional yields of the four types show little or no correlation with each other. That is, if the recombinant rh appears with a higher than average frequency in an individual burst, this is not correlated with a higher or lower than average frequency of the other recombinant type, ++, or with a higher or lower frequency of either of the parental types.

One qualification has to be made to the preceding point. If, in the yield obtained from any given bacterium, the two parental types appear with very unequal frequency, then the frequencies of both the recombinant types are lower than average. One should probably take the disparity in the yields of the two parental types as indicating that the parental type which appears with a low yield has had a late or otherwise disadvantageous start in determining what is to go on inside the



Plaques of T2h, T2r, T2rh, and T2++ on B+B/2.
T2h: small, clear; T2r: large, turbid; T2rh: large, clear; T2++: small, turbid.

bacterium. The relatively low yield of recombinants from such bacteria does not seem unreasonable under such circumstances.

5) The reciprocal cross, in which the infection is made with T2rh and T2++, has also been studied. Here the single mutants T2r and T2h appear as recombinants. Again, the two recombinants appear with equal frequency and this frequency is the same as that with which the recombinants T2rh and T2++ appeared in the original cross.

6) It will be shown below that there are several genetically distinct r mutants of T2. The principal ones studied by Hershey and Rotman

are those numbered r1, r7, and r13. When each of these is crossed with T2h, a different frequency of recombinants is obtained, as follows:

	Frequency of each recombinant
T2h x T2r1	15 %
T2h x T2r7	7 %
T2h x T2r13	0.8%

These different frequencies with which recombinants turn up are an expression of different degrees of linkage of the h locus with each of the r loci, as will be explained more fully in the section on crosses between different r mutants.

Doermann (personal communication) has carried the analysis of the h x r13 cross one step further, by breaking up the mixedly infected bacteria at a stage when the bacterium contains only relatively few infective particles. The question that Doermann wanted to decide was this: are the recombinants made by interactions stemming directly from the infecting particles, or are they made principally by interactions between their offspring? In the latter case, it should be expected that the frequency of recombinants is exceedingly low when the bacterium is opened at a time when few offspring have as yet been formed. Doermann's results show very definitely that the recombinants are represented in the early offspring with the same frequency with which they are represented in the final crop. This is perhaps the clearest evidence we have for saying that the complete phage particles formed inside the bacterium at an early stage are not themselves the parents of those which appear later.

We next turn to crosses between different r mutants of T2, say, the cross between r1 and r13 (Hershey and Rotman, 1948). Here, too, four types of particles result from the cross, namely, the two parental types T2r1 and T2r13, and the two recombinant types, T2r13 and T2++. Here, however, the three types carrying one or more r mutations form the same type of plaque. They are phenotypically alike. They can be distinguished only by genetic tests, as follows: T2r1 will give a certain proportion of wild-type progeny in a cross with T2r13, but not in a cross with T2r1r13, and of course also not in a cross with itself, which amounts to a simple multiple infection with one type. Similarly, T2r13 will give wild type progeny in a cross with T2r1, but not in crosses with T2r1r13, or with T2r13. Finally, T2r1r13 will not give wild type in any of the three possible crosses.

Such genetic tests are much more cumbersome than the simple inspection of the plaque type which was sufficient in the h x r crosses described in the previous section. In so far as they have been carried

out by Hershey and Rotman for crosses between r mutants, the genetic tests have given results which are in full agreement with the rules listed in the previous paragraph for h x r crosses. The crosses between r mutants have been useful, moreover, in throwing a little more light on linkage. Hershey and Rotman have isolated about 15 different r mutants, and have determined the frequency with which the wild type recombinant turns up in crosses between many of the possible pairs. The principal point which seems clear from the analysis is this: if a given pair yields a low frequency of wild type recombinants, this is *not* due to a generalized inability of either of these mutants to give *any* recombinations, because if either of them is crossed with a suitable third mutant, a high frequency of recombinants can be obtained from each of them.

31. *Mixed infections with unrelated strains. Interference and mutual exclusion.*—In the preceding sections we have discussed mixed infection with exceedingly closely related phages. The two parental types were derived from each other by one or two known mutational steps. We now turn to the opposite extreme, mixed infections in which the two parental types are as unrelated as possible, while yet permitting infection of the same host bacterium. The two cases best analyzed are those of mixed infection with T1 and T2 on the one hand (Delbrück and Luria, 1942), and with T1 and T7 on the other hand (Delbrück, 1945). In both cases the two parental types are morphologically distinct and show no serological cross reaction whatsoever. The principal result obtained in these cases is the phenomenon of *mutual exclusion*, which means that any one bacterium will yield upon bursting either one or the other of the two parental types, but never both. This is shown most simply and convincingly by the use of a special trick, namely plating of the mixedly infected bacteria on a mixture of two indicator strains, one of which is sensitive to one of the parental types and resistant to the other, and vice versa. On such plates, a clear plaque can be formed only from an infected bacterium which, upon bursting, yields at least one particle capable of lysing one of the indicator strains *and* at least one particle capable of lysing the other indicator strain. The frequency with which such clear plaques turn up is below 1%, the limit of reliable observation.

The principle of mutual exclusion has been verified for several other cases of mixed infection with *unrelated strains*, and no exception to this rule has yet been found.

The state of exclusion of the other virus is sometimes, but not always, very quickly established after infection. For instance, we have

seen (20) that T_5 is adsorbed in the absence of calcium but progress is arrested at an early stage. Yet, in bacteria to which T_5 has been adsorbed in the absence of calcium, the multiplication of unrelated viruses is excluded (Adams, personal communication). Similarly, in bacteria simultaneously infected with T_1 and T_2, T_1 is always excluded. Thus, T_5 and T_2 establish exclusion rapidly. On the other hand, T_1 establishes exclusion more slowly. If T_2 is added four minutes later than T_1 to a bacterial suspension, there is still a considerable proportion of bacteria in which T_2 multiplies to the exclusion of T_1.

The exclusion does *not* occur at the stage of adsorption. On the contrary, as long as the two viruses are not in very highly multiple excess of the bacteria (100 fold or more), they are each adsorbed as if the other were not there. That they are actually adsorbed to the same bacteria can be shown by a quantitative analysis of the adsorption rates and by the depressor effect described below.

When T_2 is irradiated with X-rays, its ability to exclude T_1 is destroyed at the same rate as its ability to kill bacteria. This suggests that a phage particle must invade the bacterium in order to exclude (Watson, 1950).

The same conclusion is reached by studying mutual exclusion in buffer; phage adsorbed in buffer does not exclude others added after considerable time intervals (Delbrück, unpublished). The experiments discussed in 35 indicate that phage adsorbed in buffer does not progress beyond adsorption.

In mixed infections with T_1 and T_2 exclusion is one-sided. T_2 wins out in almost every bacterium, and lysis occurs after 21 minutes, the latent period characteristic for T_2. In the few bacteria in which T_1 wins out lysis occurs after 13 minutes, the latent period characteristic for T_1. If the two phages are not given simultaneously, the time is to be reckoned from the moment at which the phage was added which is liberated by the particular bacterium.

The excluded virus, though adsorbed, is not recovered upon lysis. That its attack has actually gone beyond the stage of adsorption is indicated by its influence on the yield of the successful virus. In the case of mixed infection with T_1 and T_7, this effect of the excluded virus on the yield of the successful virus consists in a very considerable reduction of the yield. This has been called the *depressor effect.*

In the study of mixed infections with T_1 and T_7 it was found that the depressor effect itself can be mitigated by the addition of specific antiserum active against the excluded virus, added *after* adsorption of

this virus. This has been interpreted as indicating that the excluded virus remains at the outside of the infected bacterium. In view of later findings, this interpretation seems forced and the case should be restudied.

A question of great interest attaches to the possible similarity of the effects here described with the so-called interference effects observed in both animal and plant viruses. Are the interference effects in animal and plant viruses to be put into analogy with genetic recombination or with mutual exclusion or with the depressor effect? The principal point to be noted for a discussion of this question is the fact that in phages mutual exclusion and depressor effect are strongest for pairs of *unrelated viruses,* and probably *totally absent for very closely related viruses,* whereas in animal and plant viruses interference has sometimes been taken as an index of close relationship.

It should be noted that a still higher degree of unrelatedness than that here discussed could be considered, namely, that in which the two phages do not have the same host. If two such phages are added to a culture containing a mixture of the two host bacteria, presumably no interference would result. Such a case may be the analogue of the infection of a plant or an animal with two viruses attacking different tissues or, within the same tissue, attacking different components of the cell.

32. *Mixed infections with related strains* (T2, T4, T6).—Bacteria mixedly infected with two related strains, like T2 and T4, yield in the majority of cases both types of phage. If the particles of the two strains are marked with genetic markers, recombinants are found in the yield (Delbrück and Bailey, 1946), as in the cases described in 31. Beside this, type-hybrids are found in the yield (Luria, 1949), i.e., particles combining characters specific for one or the other of the parental strains, respectively.

As mentioned in 8, the main characters differentiating strains are the serological specificities and the host-range. Other strain-specific characters are the level of sensitivity to ultraviolet light and the degree of photo-reactivability. T2 and T4 are clearly separated by serological and host-range specificity, by resistance to UV (T4 being twice as resistant as T2), and by photoreactivability (T2 being considerably more reactivable than T4). The type-hybrids observed in the crosses between T2 and T4 always present the same combination of serological and host-range specificity as one or the other of the parental strains. On the other hand, their UV sensitivity may be either that of T2 or that of T4, independent of their other specificities. No intermediate levels of UV sensitivity have been found.

VII. Reactivation Phenomena

33. *Photoreactivation.*—(Dulbecco, 1949b, 1950) Phage inactivated by ultraviolet light (UV) (see 11b) is reactivated (i. e., it becomes again able to give rise to plaques) if exposed to a "photoreactivating" light under suitable conditions.

The light producing photoreactivation (phtr) belongs to the near ultra-violet and violet part of the spectrum; the action spectrum for phtr shows a band between 3,000 and 5,000 A, with a maximum around 3,650 A (Figure 3). Phtr is produced if phage *adsorbed on sensitive bacteria* is exposed to the photoreactivating light: if extracts of bacteria are substituted for bacteria no phtr occurs.

When inactive phage particles are adsorbed on washed bacteria resuspended in buffer (resting cells) their photoreactivability lasts for

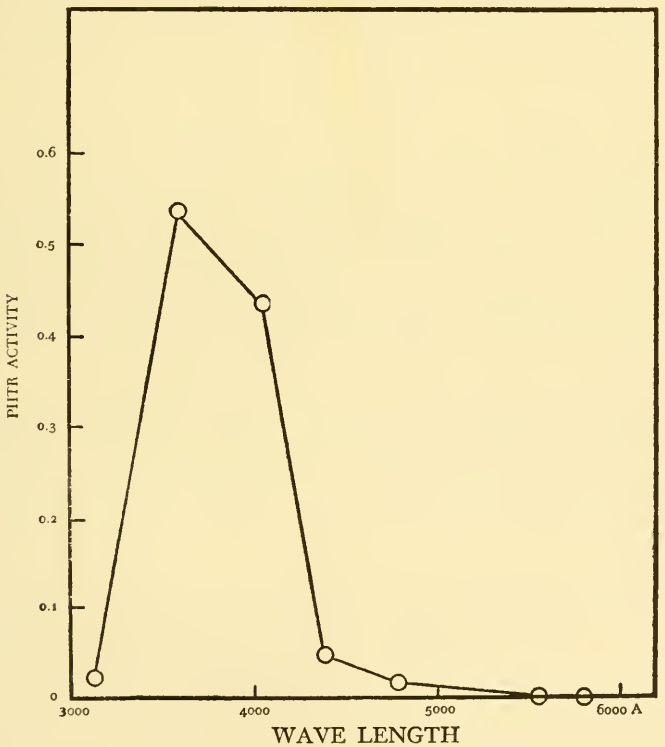


Fig. 3. Action spectrum of photoreactivation. The photoreactivating activity of light of various wave lengths, defined as being proportional to the reciprocal of the radiant energy required to produce a standard amount of PHTR, as function of the wave length.

a long time (hours); if adsorbed on metabolizing bacteria, photoreactivability ceases after 20 to 30 minutes (28°C).

After a sufficient exposure to the photoreactivating light a fraction of the inactive particles is reactivated and this fraction is not increased by further exposure to light. This divides the inactive particles into two classes, photoreactivable and non-photoreactivable ones. The relative size of these two classes is a function of the dose of UV used for inactivation. Survival curves (see 11) of the same phage in darkness and after maximum phtr have similar shape; the straight parts of these two curves (obtained at higher UV doses) make an angle. The ratio of the slope of the survival curve after phtr to the slope of the survival curve in darkness defines the "non-photoreactivable sector b of the cross-section of a phage particle for UV." The "photoreactivable sector" a=1—b is taken as an index of the photoreactivability for different phages. The phages of the T group listed in order of decreasing photoreactivability are T_1, T_2, T6, T_7, T_3, T_4, T_5. The reactivable sectors of these phages range from about 0.7 to 0.2.

Phtr as a function of the time of exposure to a constant photoreactivating light is a one-hit phenomenon: one quantum of the photoreactivating light is therefore sufficient for phtr of one phage particle. The rate of phtr is independent of the UV dose used for inactivation; it increases with the light intensity, linearly for low intensities and more slowly at higher intensities, tending to a maximum rate; the rate of phtr increases with increasing temperature, with a Q_{10} varying with the temperature from 8 near 5°C to 1.7 at 37°C.

Active phage does not show light absorption in the wave length region active for photoreactivation, but after UV irradiation of a purified phage preparation an absorption band appears in this region. The photosensitive pigment involved in the process of phtr might be this unknown substance produced in the phage or it might be a bacterial constituent.

The kinetics of the phenomenon of phtr supports the view that the reactivating light dissociates an inhibiting molecule produced by UV treatment in a phage particle, blocking some essential function, and that the dissociated inhibitor is then destroyed by a bacterial enzyme.

A very slight amount of phtr has been observed in phage inactivated with X-rays.

34. *Multiplicity Reactivation.*—(Luria, 1947; Luria and Dulbecco, 1949)

(a) Luria discovered another kind of reactivation of UV phage. It occurs when one bacterium is infected with two or more inactive particles. This is called *multiplicity reactivation* (MR). The amount of MR is defined as the *fraction of the multiple-infected bacteria that liberate active phage.* This fraction may be close to unity. The amount of MR decreases with increasing UV dose. It increases with increasing multiplicity.

Luria has proposed a theory to explain MR. According to this theory a phage particle consists of a number of specific units, the presence of one unit of each type being necessary for the formation of an active phage particle. The units are independently hit by UV radiation and one hit in one unit is sufficient for inactivating a phage particle. It is assumed that a phage particle after adsorption on the sensitive bacterium breaks up into the elementary units which multiply independently of each other in the first part of the latent period, and reassemble later, constituting the phage particles which will appear in the yield. If more than one inactive particle is adsorbed by the same bacterium, production of active phage will take place in those bacteria in which *one copy at least of each unit has not been hit by the radiation.* Quantitative formulation of this hypothesis leads to expectations which are in partial agreement with the experimental data: the deviations of the expected from the observed results can be accounted for by several possible complications.

(b) Mixed infection with two inactive particles marked at one genetic locus (Luria and Dulbecco, 1949).

In a population of bacteria mixedly infected with a very low multiplicity ($<<1$) of inactive T2r$^+$ and inactive T2r most of the mixedly infected bacteria are infected with only *one* T2r+ and *one* T2r particle, both inactive; under such conditions most of the bursts resulting from mixedly infected bacteria (due to MR) should contain T2r$^+$ together with the T2r particles. This is actually what is found. A few of the bursts are not mixed, and this is explained by the units theory by considering that in some of the infecting particles the particular unit carrying the r locus was hit by the radiation.

(c) Mixed infection with one active and one inactive particle marked at one genetic locus (Dulbecco and Luria, unpublished).

With a technique similar to that mentioned in the previous section it is possible to study the yield from bacteria infected with *one active* T2r$^+$ and *one inactive* T2r particle, and vice versa. The fraction of bursts in which the allele of the r locus of the irradiated type is not

present in the yield should measure the inactivation of the unit carrying the r locus. On this basis a survival curve (see 11) for the r locus has been determined; this curve has a curvature with a downward concavity. However, in the mixed bursts the yield of particles belonging to the same type as the radiated parent is strongly reduced *(yield reduction effect):* this shows that other factors besides the activity of the individual units interfere with reactivation.

(d) Mixed infection with one active and one inactive particle marked at two genetic loci (Dulbecco and Luria, unpublished).

The same technique is used as in the previous section. The phage combinations used were T2hr and $T2h^+r^+$, $T2hr^+$ and $T2h^+r$ (r and h not linked). Each member of each pair was used in the inactive form, combined with the other in the active form. As mentioned in 30, if a similar experiment is performed with active phages every burst contains on the average 30% recombinants. In the experiments with active and inactive phages every burst yielded the active parental type, whereas the inactive parental type appeared in a very few bursts; the recombinant types were not present in every burst, but bursts containing recombinants were much more frequent than bursts containing the parental irradiated type. Yield of phage carrying any character belonging to the irradiated parent was always strongly reduced.

VIII. UV Method for Following Early Stages of Intracellular Development

35. Bacteria which have been exposed to considerable doses of UV irradiation can still support phage growth (Anderson, 1948c). This enabled Luria and Latarjet (1947) to study the inactivation by UV of phage in the intracellular state. Bacteria are infected with T2 and incubated to the desired point in the latent period. A sample is taken, exposed to UV, and plated (before bursting). The survival of infective centers is thus determined as a function of the dose.

Immediately after infection, the survival curve obtained is approximately the same as for free phage, but as intracellular development proceeds, the resistance to UV becomes progressively much higher, although the survival curves remain exponential. About midway in the latent period, the curves develop a multiple-hit character. Similar observations, using X-rays, were made by Latarjet (1948).

The following method (Benzer, unpublished) makes use of the large change in resistance as a tool in studying phage development. If washed bacteria are infected in buffer and incubated, there is no change

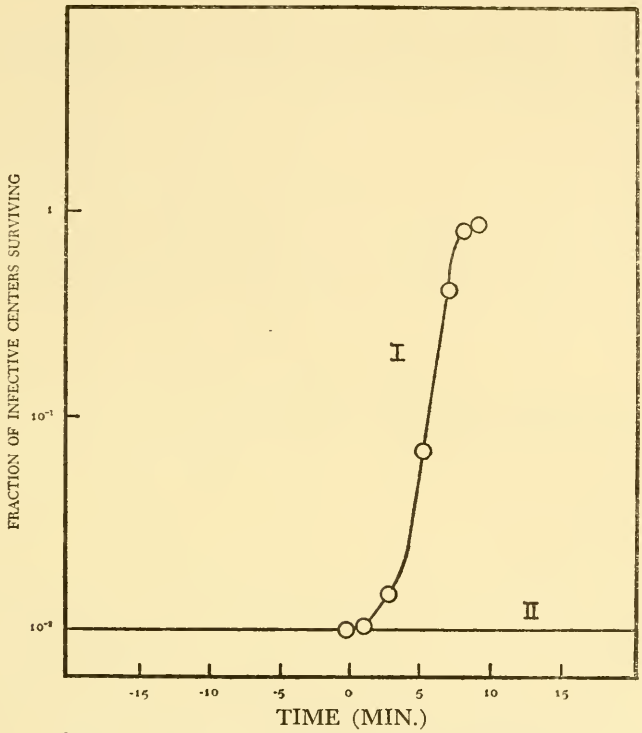


Fig. 4. Increase in radiation resistance of infective centers during intracellular development of T2r phage at 37C. The log of the fraction of infective centers surviving a constant dose of UV irradiation as function of the time subsequent to addition of broth to a suspension of infected cells in buffer (Curve I). A control is shown to which no broth has been added (Curve II).

in radiation resistance of infective centers with time. Suppose a standard dose is chosen which gives a survival of 1%. Then, as long as no nutrients are supplied and intracellular development thus does not take place, the survival of a sample subjected to the standard dose continues to be 1%. If broth is added at any time, the survival begins to rise in the same manner as observed if the infection is initially made in broth. (See Figure 4.) The adsorption time of the phage is effectively reduced to zero in this way, since all infective centers start to develop at the same time. After seven minutes in the presence of broth at 37°C, the survival becomes 40 times as high as initially for the same dose.

The radiation resistance of an infective center is thus a very sen-

sitive index to development in the earliest stages of the latent period, at a time before the completion of viable new phage particles, and the technique is being applied to several problems.

For example, the nutritional requirements for synthesis of phage can be studied by starting in buffer and adding well-defined nutrients. If the added materials are inadequate, no change in resistance occurs. If completely adequate, the rate of increase should correspond to that observed with broth. In cases where chemical transformation and adaptive enzyme formation are necessary, these will be revealed by the shape of the curve.

IX. Cytology

36. Observations on *E. coli* bacteria have been made with the staining method of Piekarski-Robinow. Before the infection one sees usually two or four colored bodies, "nuclei," inside the cell. Five minutes after infection with T2 the nuclei seem to break down into masses that line the periphery of the cell. Later, a granular material appears and progressively fills the cell (Luria and Human, 1950).

In the dark field microscope the live uninfected bacterium appears as a cigar shaped body whose outline only is visible. The interior does not scatter enough light and thus appears completely dark. This is still true of infected bacteria; however, they are marked by the presence on their surface of strongly scattering dots and the number of these dots seems to be similar to the multiplicity of infection. Later, the inside of the bacterium fills with small particles in violent motion. Bursting of the bacteria may take place in various manners. The small particles inside may congregate near the middle of the bacterium which swells to a spherical shape and then explodes (the whole process taking less than a second) scattering hundreds of particles around itself, these phages slowly diffusing through the preparation. A long bacterium may break in two, one half hinged to the other, slowly folding back against the other while the phages quickly ooze out of the opening. Sometimes the infected bacterium will swell to spherical shape failing to explode, never liberating phage.

There is no doubt (see 4) that the particles which are to be identified with the phages emerge from the *inside* of the bursting bacterium (Weigle, unpublished).

X. Lysogenesis

37. The term "lysogenic strain" has been applied to those bacterial strains in cultures of which the presence of bacteriophage can be detected. If a culture, or the filtrate of a culture of a strain originat-

ing from a single colony, can form plaques when plated on one of several bacterial strains, the culture is said to be lysogenic. The strains sensitive to the action of the phage carried by the lysogenic bacteria are called *indicator strains*, and can be used to prepare stocks of this phage.

When systematic searches of this kind have been carried out, a large proportion of the bacterial strains examined, whether freshly isolated or taken from laboratory collections, were found to be lysogenic. It is furthermore impossible to ascertain whether a strain which did not give plaques on the indicator strains employed would not have proved to be lysogenic if a *suitable* indicator strain had been available. Such findings have been made with a number of genera (sporulated soil bacteria, salmonellae, pseudomonas, vibrio, corynebacteriae, staphylococci, etc.). Lysogenic strains, in fact, seem to be the main reservoir of bacteriophages in nature.

The above definition, although widely accepted, is too ambiguous to permit an understanding of the underlying factors involved. The equilibrium between the development of bacteria and the reproduction of phage can, in effect, be one of two types of phenomena of obviously different significance. On the one hand, it can be a population equilibrium involving external phage and the whole bacterial population. Such strains may be called *carrier strains*. On the other hand, the equilibrium may exist at the intra-cellular level. The term *"true" lysogenic strain* should be reserved for this second case.

Carrier Strains. The *carrier strains* do not represent a phage-host relation of fundamental difference from those discussed extensively in previous sections. Such strains can always be freed from phage, and thus lose their lysogenicity, by methods which inactivate free phage or prevent its adsorption to the host cell. The apparent lysogenicity can be recognized to be due to either one of two principal reasons:

(1) The bacterial cells are sensitive to the action of the phage, but in a culture, uninfected cells outgrow virus reproduction. This may be due to such factors as poor adsorbability, long latent period, low burst size, etc.

(2) The great majority of the bacterial cells are resistant to the action of the phage but may adsorb it reversibly, i. e., "carry" it. Multiplication of the phage occurs by lysis of sensitive mutants arising in the culture.

"True" Lysogenic Strains. True lysogenic strains cannot be freed

from phage by means which inactivate free phage or prevent adsorption to the host cell.

Among strains which comply with this definition and which have been most carefully studied are the strain of *E. coli* of Lisbonne and Carrere (Bordet and Renaux, 1928) and the *B. megatherium* 899 (Den Dooren de Jong, 1931; Wollman and Wollman, 1938; Lwoff, 1950).

For the case of *B. megatherium* 899, the following facts have been found:

(1) When equal aliquots of a carefully washed suspension of the lysogenic strain are plated for plaque formation on the indicator strain, on the one hand, and for colony count, on the other hand, an equal number of plaques and colonies is found.

(2) The progeny from each spore (even when heated to temperatures which destroy free phage) and from each bacterium is a true lysogenic strain.

(3) A culture remains lysogenic even after serial transfers in medium containing anti-phage serum.

(4) Even though calcium ions are required for the propagation of the megatherium phage on the sensitive strain, serial transfers of the lysogenic strain in calcium-free medium leave its lysogenic function unimpaired.

(5) Isolated bacteria cultivated under the microscope can undergo up to 19 divisions without release of phage to the medium, and the progeny is still lysogenic. Lysis of isolated bacteria can take place without production of free phage, but appearance of phage in the medium is always preceded by the lysis of a cell.

(6) If a washed suspension of lysogenic bacteria is artificially lysed by means which are known not to affect free phage (lysozyme), no phage is released to the medium.

These findings show that virus reproduction in true lysogenic strains differs conspicuously from that observed with T-phages and their *E. coli* host, as described in previous sections. Each single bacterial cell appears to be capable of carrying the potentiality for reproduction of the virus *without further intervention of external phage* while at the same time undergoing the processes of assimilation and multiplication. The fact that there may be lysis, either spontaneous or induced, in which no infective particles are released to the medium,

suggests that, as in the early stages of the latent period (see 27), the phage is present in the cell in a non-infective state.

XI. Lysis Inhibition

38. Phages T2, T4, and T6 differ from their r mutants (see 8) in that the r mutants produce clearing of turbid bacterial cultures at the end of the latent period while in cultures infected with the wild type r^+ clearing is considerably delayed, sometimes for hours (Hershey, 1946b). This delay of clearing, which is characteristic for *turbid cultures* of bacteria infected with the *wild types* of the even numbered phages of the T set, is called *lysis inhibition*, and the letter *r*, characterizing the mutants in which the delay is absent, stands for rapid lysis. The difference between r and r^+ has been analyzed by Doermann (1948b) as follows:

(1) Lysis inhibition is due to a second infection with phage coming at least 3 minutes after the primary infection. The r^+ phages liberated from the first lysed bacteria are adsorbed by the remaining bacteria and inhibit their lysis.

(2) To inhibit lysis both primary and secondary infection must be with r^+ phage.

(3) While one particle can inhibit lysis, the duration of the inhibition increases with the multiplicity of the secondary infection.

(4) The burst size of lysis inhibited bacteria is greater than that of uninhibited bacteria.

(5) T2, T4, and T6 can cross inhibit lysis, e.g., following primary infection with $T2r^+$, a second infection by $T4r^+$ results in lysis inhibition.

The following observations suggest that inhibition does not involve growth of the secondarily added phage:

(1) When a bacterium is primarily infected with $T2h^+r^+$ and 8 minutes later with $T2hr^+$, lysis inhibition occurs though none of the inhibited bacteria yield any $T2hr^+$ (DeMars, personal communication).

(2) "Non-killing" X-ray inactivated $T2r^+$ (see 21) inhibits lysis as effectively as does active $T2r^+$. This suggests that inhibition is due to a stage prior to invasion and may be due to a change in the bacterial surface (Watson, 1950).

Nothing is known about the chemical mechanism of lysis inhibition.

XII. Lysis from Without

39. When a large number (100 or more) of phage particles are adsorbed to a bacterium, no phage multiplication occurs. Instead, the bacterium quickly becomes spherical and eventually lyses (Delbrück, 1940b). Since this lysis occurs without phage growth it has been called *lysis from without* (LFW) in contrast to normal lysis. More recently LFW has been observed under the following additional conditions:

(1) Infection of a bacterium by only a few phage particles in the presence of cyanide, iodoacetate, or dinitrophenol results in LFW (Cohen, 1949; Doermann, 1948; Heagy, 1950). Lack of oxygen or of a carbon source also causes LFW. This suggests that LFW occurs *at low multiplicities* when a suitable energy source is absent. Bacteria heavily irradiated with UV lyse prematurely when *singly infected* (Anderson, 1945b). This may also be due to a disruption of the normal energy supply by the UV irradiation. The r+ phages are more effective than the r phages in inducing LFW in the presence of metabolic inhibitors (Cohen, 1949; Heagy, 1950). The speed at which LFW occurs (measured by decrease in bacterial turbidity) increases with the multiplicity of the infection (Watson, 1950).

(2) LFW occurs without disruption of the bacterial nuclei (see 36). This is further evidence that LFW is independent of phage growth (Watson and Human, unpublished). Furthermore, "non-killing" X-ray inactivated phage (see 21) can cause LFW (Watson, 1950). Therefore LFW is not due to invasion of the phage into the bacteria.

(3) "Non-killing" X-ray inactivated T2 is much more effective than active T2 in causing LFW (Watson, 1950).

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	T2	T4	T6	T5	T1	T3	T7
Serological Grouping See §7							
Size and Shape x 40,000							
Minimum Latent period in broth at 37C (minutes)	21	24	25	40	13	13	13
Plaque Size	small	small	small	small	medium	large	large
Physical Characteristics	Sensitive to Sonic Treatment and Osmotic Shock			Sensitive to sonic treatment	Stable when dry		
% adsorbed/min on 5 x 10^7 B/ml in broth at 37 C	20	20	20	1	10	10	10
Sensitivity to UV. Dose in 10^3 Erg/CM^2 for 10^{-3} survival	7	14	7	9.3	44	53	53
Photoreactivable Sector (see §33)	.56	.20	.44	.20	.68	.39	.35
Multiplicity Reactivation (see §34)	++++	++++	++++	++	+	-	-
Principal Mutants	h,r (several)	r(several), cofactor	r, cofactor		h	h	h

TABLE I. Properties of the T Phages